



Regulation of Genome-Wide Transcriptional Stress Responses in *Saccharomyces cerevisiae*

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**Regulation of genome-wide transcriptional stress responses in
*Saccharomyces cerevisiae***

Abstract

In response to osmotic shock in *Saccharomyces cerevisiae* the MAP kinase Hog1 coordinates a large-scale transcriptional stress response, rapidly producing hundreds of copies of specified transcripts. Many of the most highly induced genes are bound and regulated by a transcription factor, Sko1, but lack the canonical binding site for this factor. We use ChIP-seq to demonstrate a stress-specific binding mode of Sko1. In stress, Sko1 binds to promoters in close proximity to Hog1, and another Hog1-regulated transcription factor, Hot1. This mode of Sko1 binding requires the physical presence of Hog1, but not Hog1 phosphorylation of Sko1. We identify candidate Sko1 and Hot1 binding motifs that predict co-localization of Sko1, Hot1, and Hog1 at promoters. We then demonstrate a role for Sko1 and Hot1 in directing Hog1-associated RNA Pol II to target genes, where Hog1 is present with the elongating polymerase. We suggest a possible model for Hog1 reprogramming of transcription in the early stages of the osmotic stress response. We then determine the extent and structure of the Hog1 controlled transcriptional program in a related stress, damage to the cell wall. We find that Sko1 and Hot1

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have different apparent thresholds for activation by Hog1. In addition, in cell wall damage, Hog1 regulates an additional transcription factor, Rlm1, that is not involved in other Hog1 regulated stress responses. This factor is activated by the coincidence of a signal from Hog1 with that of another MAP kinase, Slt2.

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Statement of contributions

Professor Erin K. O'Shea (EKO) supervised all work.

Chapter 1

Kristen E. Cook (KEC) wrote the text.

Chapter 2

KEC performed the experiments, analyzed the data, and wrote the text.

Chapter 3

KEC performed the experiments, analyzed the data, and wrote the text.

Chapter 4

KEC performed experiments, analyzed the data, and wrote the text.

EKO and Andrew Capaldi assisted in the design of the experiments and with an early draft of the text.

Ying Liu performed microscopy experiments.

Chapter 5

KEC wrote the text.

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Chapter 1

Introduction to stress-induced transcriptional regulation by the MAP kinase Hog1 in *Saccharomyces cerevisiae*

Introduction to transcriptional responses to environmental stress

As cells grow and divide, they must sense and respond to changes in the environment. A cell's ability to survive an unexpected environmental challenge, such as heat, oxidative damage, or a change in osmolarity, depends upon the set of proteins already at hand (Proft and Struhl, 2004; Mettetal et al., 2008; Westfall et al., 2008). Because of this, the signaling mechanisms that allow initial survival of these stresses tend to be post-translational. Transcriptional responses are not the front line of defense against stress; instead, they are strategies for ensuring that cells are able to repair damage, adapt to the changed environment and continue growth. These transcriptional responses are clearly important; cells devote considerable resources to transcriptional stress responses. In the budding yeast *Saccharomyces cerevisiae*, these responses can alter the expression levels of thousands of genes within minutes (Gasch et al., 2000).

Yeast cells regulate the expression levels of over 6000 genes across a variety of conditions, using fewer than 200 transcription factors to communicate the specific program for each gene. Although many of the signaling components, and connections between these components, that regulate transcription have been identified, several questions remain. We do not understand how these signals are read out at individual promoters to set a precise expression level for each gene. For example, are some signals interpreted differently depending on which other signals are present? If so, at what point in the signaling process is this decision made? It is not clear how much of this information processing work is performed by

transcription factors. Can transcription factors themselves integrate signals, and is this a common strategy? Can the same transcription factor function both to amplify signals, and to faithfully transmit signals without distortion?

There are over 6,000 genes in the *Saccharomyces cerevisiae* genome, but most mRNAs are only present at the level of a few copies per cell (Holstege et al., 1998). However, large-scale shifts in the identity and abundance of mRNAs present in the cell occur rapidly in response to stress, when genes that were previously unexpressed are transcribed hundreds of times within minutes (Romero-Santacreu, 2009). It is not clear if these large-scale changes in gene expression levels require special mechanisms. Can the same signal that specifies moderate induction of one set of genes also convert an unexpressed gene into the most highly transcribed gene in the genome? Perhaps these two tasks require different signaling mechanisms. Beyond considerations of regulation at individual promoters, it is not clear if cells have the transcriptional resources to carry out both a standard gene expression program and a stress responsive program simultaneously. This question has rarely been posed, perhaps because the extent and magnitude of environmental stress responses were difficult to quantify before genome-wide data sets became widely available. However, the popularity of genomics approaches to studying stress responses in the last ten years has made these types of questions more tractable.

The scale of transcriptional stress responses

Based on measurements of mRNA abundance under standard growth conditions (Ingolia et al., 2009; Yassour et al 2009) and microarray measurements of fold-change in gene expression during stress (e.g. moderate osmotic shock; Capaldi et al, 2008), it is possible to estimate the actual number of transcripts produced during a stress response. (Recent work (Miller et al., 2011) attempted a direct measurement of the absolute levels of mRNA produced during this stress response, but a lack of a normalization scheme makes it difficult to compare values obtained under different conditions.) By this logic, a few hundred copies per cell of the most highly induced individual transcripts are produced in a span of twenty minutes, suggesting that the average cell produces about 10 copies of each of these transcripts per minute during stress. This is about twice the highest rate of production of the most highly expressed genes in the absence of stress; Miller and colleagues (2011) report that the most highly expressed transcripts (those encoding ribosomal proteins, cell wall proteins, and enzymes involved in fermentation) are produced at a rate of about 4 per minute per cell during rapid growth in rich medium. It is not clear whether cells in rapid growth are operating near their maximum transcriptional capacity, or what sets this maximum. For example, it is not clear if all RNA Pol II holoenzyme components are present in excess.

Perhaps the reason stressed cells seem to exceed the maximum pre-stress transcriptional rate for a single promoter is because the population is effectively synchronized by stress. Before stress, a population of rapidly growing cells is

relatively heterogeneous, with different cells at different stages of the cell cycle. Any given transcript may rarely be at its full expression level in 100% of the population at a single point in time. The population-wide maximal transcription rates observed in stress may be more representative of true transcription rate of highly expressed genes than the rates than can be observed in happily growing cells. Studying large-scale changes in transcriptional programs provides an opportunity to learn more about the general properties and limits of transcriptional regulation.

The HOG Pathway in Osmotic Shock

The osmotic shock response coordinated by the high osmolarity glycerol (HOG) pathway in budding yeast is one of the best characterized and most tractable examples of a large-scale transcriptional change. This response rapidly induces the expression of hundreds of genes, and is coordinated by the MAP kinase Hog1 (Figure 1.1). In response to an increase in osmolarity, two separate membrane-associated sensors are activated, and these signals converge upon the MAP kinase kinase Pbs2 (Posas et al., 1996; Posas and Saito, 1997). Pbs2 phosphorylates the MAP kinase Hog1 (Brewster et al., 1993), triggering translocation of Hog1 to the nucleus (Ferrigno et al., 1998). The stress-induced transcriptional program involves the action of at least four transcription factors, some of which act redundantly (Capaldi et al, 2008). These four factors are: the general stress transcription factors Msn2 and Msn4 (Rep et al., 2000), which may or may not be directly regulated by Hog1; and two factors Sko1 (Proft et al., 2001; Proft and Struhl, 2002) and Hot1

(Rep et al., 2000; Alepuz et al., 2001; Alepuz et al., 2003) that are known phosphorylation targets of Hog1.

Hot1 targets a small set of highly induced genes (Rep et al., 2000; Alepuz et al., 2001; Alepuz et al., 2003; Capaldi et al., 2008), and its binding and activity both require Hog1 (Alepuz et al., 2001; Alepuz et al., 2003). While Hog1 phosphorylates Hot1 upon stress, this phosphorylation is not required for Hot1 activity (Alepuz et al., 2003). Instead, Hog1 is physically present with Hot1 as a required binding cofactor at target promoters, and it is Hog1 that recruits the polymerase (Alepuz et al., 2003). The role of Hot1 is to direct Hog1 to appropriate genomic locations, where it can promote transcription by interacting with general transcription machinery.

While Hot1 regulates gene expression at a small class of genes during stress, Sko1 affects a larger set of target genes, and is regulated by Hog1 under conditions of rapid growth as well as stress. Sko1 is a member of the cyclic AMP response element binding (CREB) family of transcription factors (Nehlin et al., 1992; Vincent and Struhl, 1992). Like other CREB factors, Sko1 contains a basic leucine zipper domain that allows dimerization and DNA binding. The CREB factors share DNA binding specificity for the palindromic motif TGACGTCA, and Sko1 recognizes this site both *in vivo* in the absence of stress (Harbison et al., 2004; Capaldi et al., 2008) and *in vitro* (Nehlin et al., 1992; Fordyce et al., 2010). Under conditions of rapid

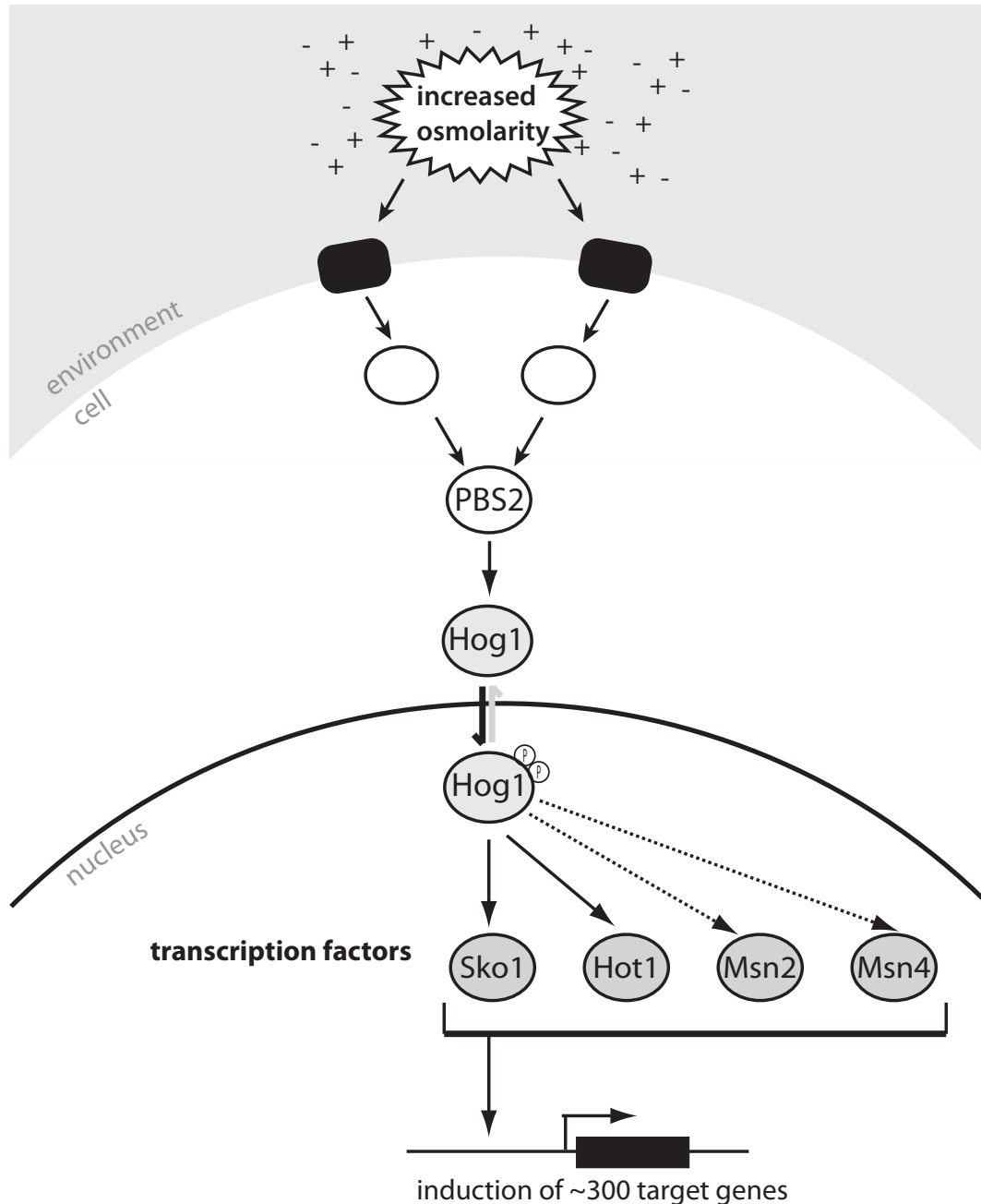


Figure 1.1. Activation of the HOG pathway transcriptional response in stress

In response to increased osmolarity, signals from two separate sensors converge on the MAP kinase kinase Pbs2, resulting in the phosphorylation of Hog1. Phosphorylated Hog1 translocates to the nucleus, where it activates its cognate transcription factors Sko1 and Hot1. In addition, Hog1 may play a role in activating the general stress factors, Msn2 and Msn4. Together, these transcription factors coordinate the induction of hundreds of genes.

growth, Sko1 binds at the promoters of at least 100 genes (Capaldi et al., 2008) and represses the transcription of a subset of these genes by recruiting a co-repressor complex, Tup1-Ssn6 (Proft and Struhl, 2002). The repressive activity of Sko1 appears to be regulated both by PKA (Nehlin et al., 1992; Pascual-Ahuir et al., 2001) and by Hog1 (Proft et al., 2001; Proft and Struhl, 2002).

PKA phosphorylates Sko1 at three positions, and PKA activity is required for the nuclear localization of Sko1 (Pascual-Ahuir et al., 2001). In addition, phosphorylation by PKA moderately increases Sko1's binding affinity for the CREB motif *in vitro* (Pascual-Ahuir et al., 2001). In general, PKA phosphorylation of Sko1 seems to be associated with repression, while Hog1 phosphorylation results in relief of repression (Proft and Struhl, 2002). Under conditions of rapid growth, basal Hog1 activity restrains repression by Sko1 (Proft and Struhl, 2002, Capaldi et al., 2008). These genes are further repressed in strains lacking Hog1. Overall, in the absence of stress, Sko1 binds to a well-defined sequence element, represses genes by recruiting repressive binding partners, and is regulated by phosphorylation.

In stress, Sko1 displays remarkably different properties. In response to osmotic stress, Sko1 activates a subset of its repression targets, as well as a set of genes that were not previously bound or regulated by Sko1 (Proft and Struhl, 2002; Capaldi et al., 2008). At some promoters, this activation may involve the recruitment of additional, activating chromatin modifiers to the existing repressive

complex (Proft and Struhl, 2002), although the extent of this activation mechanism is not clear. Specifically, there is no evidence suggesting that such a complex assembles at Sko1 activation targets that were not bound by Sko1 in its pre-stress repressive mode. However, it is clear that activation by Sko1 requires Hog1. In stress, Hog1 both phosphorylates Sko1 and co-localizes to some Sko1-bound promoters (Proft and Struhl, 2002; Capaldi et al., 2008). Interestingly, Sko1 binding increases at its activation targets in stress, although it is depleted from the promoters of some repression targets (Capaldi et al, 2008). In stress, there is a shift both in the binding preferences of Sko1 and in the proteins it associates with at promoters. Neither of these changes is well understood.

Hog1 regulation of its two cognate transcription factors involves both stoichiometric and catalytic signaling mechanisms. Hog1 regulates Sko1 enzymatically, via phosphorylation. A very small amount of nuclear Hog1 is able to modulate Sko1 activity by this mechanism. Hog1 regulation of Hot1, and possibly Sko1 in stress, requires stable physical interaction between stoichiometric amounts of these proteins. Investigating the possibility of catalytic *versus* stoichiometric actions of Hog1 in regulating these factors may shed light on the general role of these two methods of regulation in signaling.

Hog1 as a global regulator of transcription during stress

In the first few minutes following osmotic shock, transcription rates globally decrease (Proft and Struhl, 2004; Romero-Santacreu et al., 2009; Miller et al., 2011).

Specifically, transcription initiation rates drop, apparently due to decreased association of general and specific transcription factors with genomic DNA factors (Proft and Struhl 2004). This effect is thought to be caused by increased ionic strength of the nucleoplasm due to a contraction of volume. These physical effects inhibit transcription initiation in the first few minutes of stress, although elongating polymerase complexes are not affected, and transcription factor binding resumes by five minutes after stress treatment. However, the global decrease in transcription rate upon osmotic shock is about half as severe in strains lacking Hog1 (Romero-Santacreu et al., 2009). This result suggests that the physical effects of stress are not the sole cause of the observed global decrease in transcription, and that HOG pathway signaling may be partially responsible triggering this decrease. This result points to a general role for Hog1 in globally modulating transcription upon stress, beyond the targeted activation of the approximately 300 (Capaldi et al., 2008) Hog1 induced genes.

Over the last several years, Hog1 has been implicated in a growing list of interactions that suggest mechanisms by which Hog1 could globally regulate transcription or chromatin state during stress. Several Hog1 interactions with chromatin modifying enzymes have been reported. Hog1 is thought to recruit the coactivators SAGA and SWI/SNF to some Sko1 regulated genes (Proft and Struhl, 2002). Hog1 also interacts with the histone deacetylase Rpd3 upon stress, targeting this chromatin-modifying enzyme to specific promoters (de Nadal et al., 2004). Stress-induced chromatin remodeling has also been shown to depend upon the interaction between Hog1 and the RSC complex (Mas et al., 2009). In addition,

Hog1 regulates the ubiquitin ligase Ubp3 in a stress-dependent manner, and Ubp3 associates with stress induced genes and is required for their expression (Solé et al., 2011). At some genes (e.g. *STL1*, a popular reporter gene for osmotic stress induction), nearly all of these interactions are thought to occur. However, no current model addresses the combined action of these chromatin regulatory mechanisms, and there is no specific evidence for Hog1 regulation of chromatin remodeling beyond the set of stress responsive genes.

Hog1 also associates with general transcriptional machinery during stress. In addition to its role in recruiting the polymerase to Hog1 target genes (Alepez et al., 2003) during transcription initiation, Hog1 associates with elongating RNA Pol II during stress. Under osmotic shock conditions, the MAP kinase Hog1 forms a complex with RNA Pol II and is present in some ORFs during elongation (Proft et al., 2006). At these genes, Hog1 is part of the elongating RNA Pol II holoenzyme complex. Hog1 interacts with the major subunit of the polymerase *in vitro* (Proft et al., 2006), although the affinity of this interaction has not been measured. *In vivo*, Hog1 is excluded from the nucleus in absence of stress, which would prevent this interaction from occurring, but in stress, the concentration of nuclear Hog1 increases dramatically within minutes (Ferrigno et al., 1998, Capaldi et al., 2008), which may allow Hog1 to stably associate with a fraction of RNA Pol II holoenzyme complexes during stress. Hog1 has been observed in the ORFs of several stress-induced genes by chromatin immunoprecipitation (ChIP) during osmotic shock, and this result is robust to the choice of tag for the pulldown (Proft et al., 2006; Pokholok et al., 2006).

The reported physical interactions between Hog1 and RSC, SAGA, SWI/SNF, Rpd3, Ubp3, and RNA Pol II are likely to play an important role in the activation of Hog1 target genes during stress. However, the ever-increasing list of interactions between Hog1 and components of general transcriptional machinery or general chromatin modifiers poses additional questions about the role of Hog1 in stress, rather than explaining previously observed behavior. The observation that a stress induced, transient global decrease in transcription depends upon Hog1 (Romero-Santacreu et al., 2009) suggests that Hog1 globally regulates the allocation of transcriptional resources during the induction phase of the stress response. However, none of the reported interactions between Hog1 and general transcription machinery or chromatin-remodeling enzymes have ever been shown to have a genome-wide effect. Many questions remain about the role of Hog1 regulation of general transcriptional regulators. Most generally, the canonical model of condition specific transcriptional activation in eukaryotes is that signaling kinases modulate the localization and/or activity of cis-acting, gene-specific transcription factors, which then recruit transcriptional machinery to appropriate genes. Models of regulation in which the signaling kinase itself, in this case Hog1, interacts with global regulators, and is physically present at target genes during transcription do not fit these expectations, and are not well explored.

When Hog1 interacts with a global regulator, such as RNA Pol II, it is not clear if the influence of this interaction limited to small subset of genes, as suggested by previous work (Proft et al., 2006; Pokholok et al., 2006), or how these gene targets

are specified. Elucidating the mechanisms and gene-specificity of interactions between Hog1 and globally important transcriptional components will help clarify the extent and function of this type of regulation.

Beyond osmotic shock: Hog1 as a general stress signal

Hog1 is also thought to play a role in responding to several environmental stresses in addition to high osmolarity. The Hog1 pathway is necessary for stress tolerance and the induction of specific genes across several different stress conditions, including damage to the cell wall (Alonso-Monge et al., 2001), low pH (Kapteyn et al., 2001; Lawrence et al., 2004), decreased temperature (Pandero et al., 2006), increased temperature (Winkler et al., 2002), and oxidative damage (Singh, 2000). The HOG pathway is active in a diverse set of conditions, including conditions which may impose opposite demands on the cell (e.g. heat *versus* cold). Because of its role in a diverse set of conditions, the HOG pathway may provide a useful model for studying condition specific output by MAPKs. However, previous genomic studies of Hog1 function have focused almost exclusively on its role in osmotic stress. Although Hog1 is active in several conditions, it is not clear if Hog1 activates a stress-specific gene expression program in each of these conditions. One detailed study of HOG pathway activity in two different osmotic shock conditions suggests that this pathway is capable of stress-specific output that varies based on the presence of other stress-activated signals (Capaldi et al., 2008). When yeast cells are exposed to moderate osmotic shock with either salt or glucose as the osmolyte, Hog1 is activated to similar levels, and coordinates a core osmotic shock response.

However, in osmotic shock with salt as the osmolyte, a set of genes in addition to this core response is regulated by the Hog1 pathway through the action of the general stress factors Msn2 and Msn4. For full activity, these transcription factors require the coincidence of active Hog1 with an independent, osmolyte-specific signal (putatively PKA). In this way, Hog1 signaling is context dependent, i.e. active Hog1 is interpreted differently by target factors depending on which other signaling pathways are active.

Recent work suggests that a combination of signals between the HOG pathway and cell wall integrity pathway (also a MAP kinase cascade) is required for the transcriptional response to cell wall damage (Bermejo et al., 2008; Garcia et al., 2009). It will be interesting to see how the Hog1 dependent response to cell wall damage differs from the Hog1-dependent osmotic shock response, and if these differences can be attributed to context-dependent signaling in the Hog1 pathway.

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Chapter 2

Investigation of binding determinants of the CREB family transcription factor Sko1

Introduction

In response to osmotic stress, a transcriptional network controlled by the MAP Kinase Hog1 activates over 300 genes (Capaldi et al., 2008). Expression of this stress response program primes cells to respond more quickly, should they face a similar stress in the near future (Mettetal et al., 2008). Four transcription factors control activation of the Hog1 target genes: the general stress factors Msn2 and Msn4, as well as Sko1 and Hot1, which interact directly with Hog1 (Alepuz et al., 2001; Proft and Struhl, 2002). Sko1 and Hot1 are especially important in promoting transcription at the most highly induced genes in stress, and these two transcription factors tend to act at the same genes, although Sko1 has more targets. Sko1 influences the expression of 54 genes in stress (Capaldi et al., 2008).

Sko1 is a cyclic AMP response element binding (CREB) family transcriptional regulator that can act as both an activator and a repressor (Proft and Struhl 2002, Capaldi et al., 2008). Sko1 is localized to the nucleus and is phosphorylated by at least three different kinases: Hog1, PKA, and Sch9 (Pascual-Ahuir and Proft 2007; Proft et al., 2001). The repressive activity of Sko1 is better understood than its role in activation. Under favorable growth conditions, Sko1 inhibits gene expression by recruiting the Tup1-Ssn6 co-repressor complex to the promoters of target genes. This repression is relieved by phosphorylation of Sko1 by the MAP kinase Hog1, which is activated by high osmolarity, as well as other stresses.

At individual genes, Sko1 can act as either a repressor or activator, or both, depending on cellular conditions. Previously, Sko1 was thought to be constitutively bound to its target promoters, and depending on cellular conditions, recruit either repressive or activating chromatin remodeling factors (Proft and Struhl, 2002). In this model, Sko1 serves as a marker of certain cis-elements, and directs other condition-specific regulators that lack specific DNA binding affinity to these elements. However, a genome-wide study of Sko1 binding behavior demonstrates that Sko1 target promoters do not display uniform binding behavior. Instead, genes with Sko1 bound in the promoter fall into two basic classes (Capaldi et al., 2008). At class I genes, Sko1 is bound in normal growth conditions and released from the promoter in response to osmotic stress. At class II genes, Sko1 binding enrichment increases in response to stress. A subset of class II genes are not bound or regulated by Sko1 in control conditions; Sko1 acquires these genes as binding targets upon stress. Most of the genes that are activated by Sko1 in stress display class II binding behavior.

We are interested in understanding what causes differences in Sko1 binding behavior at different genes. Specifically, how does Sko1 binding decrease at some promoters and simultaneously increase at others? In this study, we use ChIP-seq to generate a high resolution map of Sko1 binding, and are able to classify individual binding events as class I or class II. We identify specific cis-regulatory elements that are specific to class I and class II binding behaviors. We identify stress specific factors that co-localize with Sko1 at class II binding sites, and propose a model of stress-specific occupation of class II binding sites.

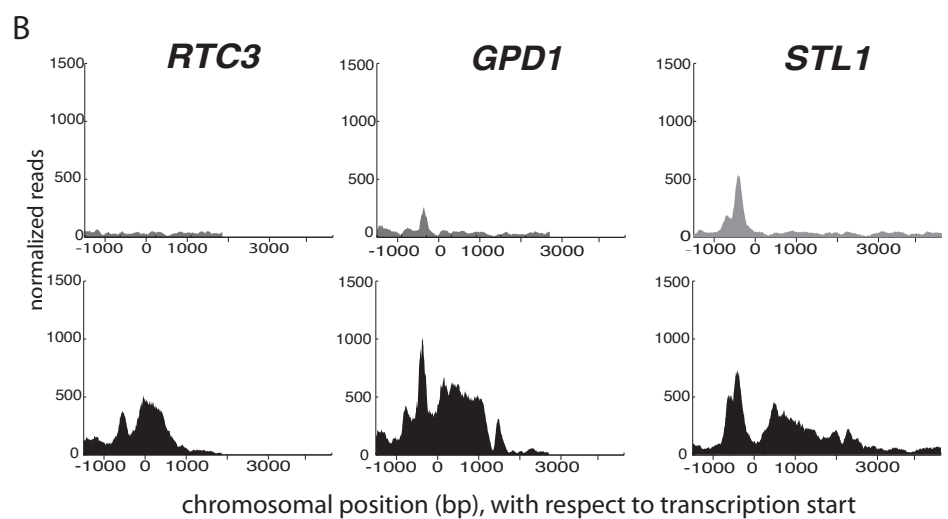
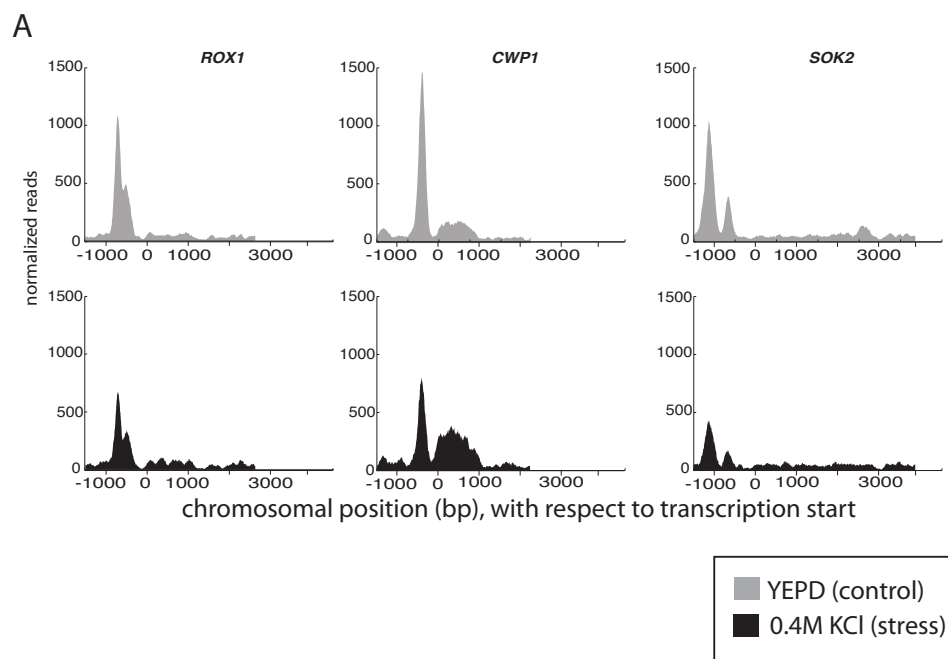
Results

ChIP-seq allows identification of class I and class II binding events

Many Sko1 bound promoters seem to be bound at multiple sites, based on the shape of ChIP-chip enrichments peaks (Capaldi et al., 2008). However, the resolution of the existing data is not high enough to resolve the locations of nearby peaks, or identify differences in binding behavior between them. To determine more precisely which cis elements are recognized by Sko1 under both normal growth conditions and during osmotic shock, we performed ChIP-seq on a strain expressing HA-tagged Sko1. We then selected the 100 peaks with the highest enrichment over input samples for farther analysis (listed in Table 2.S1). Individual binding peaks within promoters generally display either class I or class II binding behavior. At class I binding peaks, Sko1 is highly enriched during logarithmic growth in YEPD (Figure 2.1A, gray plots). Upon stress, Sko1 enrichment at these peaks decreases (Figure 2.1A, black plots). At class II binding peaks, Sko1 enrichment is greatest during stress (Figure 2.1B, black plots), and enrichment is reduced or absent under control conditions (Figure 2.1B, gray plots). At genes that exhibit class II binding, Sko1 is often enriched in coding regions as well as regulatory elements. This signal may be due to a strong interaction with Hog1, which physically associates with RNA

Figure 2.1. Two classes of Sko1 binding sites observed by ChIP-seq. Sko1 binding was measured in control conditions as well as after five minutes of exposure to osmotic shock, which was induced by addition of 0.4M KCl to the medium. Regions of genomic DNA that associate with Sko1 were identified by ChIP-seq against Sko1 tagged with three copies of the HA epitope. Sko1 binding in the control condition is shown in gray; binding in stress is shown in black. **(A)** Class I binding peaks are present in both control and stress conditions. **(B)** Class II binding peaks are substantially increased in enrichment upon stress. At some class II peaks, no Sko1 enrichment is observed in the absence of stress (eg *RTC3*). At other class II peaks, binding occurs in the absence of stress, but is enhanced upon stress treatment (e.g. *STL1*)

Figure 2.1 continued



Pol II and travels with the polymerase during elongation (Proft et al., 2006, see discussion in Chapter 2.) This possible (indirect) interaction between Sko1 and RNA Pol II is unlikely to affect many RNA Pol II complexes, given that Sko1, like most transcription factors, is not present in high abundance.

Class I binding occurs at the known Sko1 consensus binding site

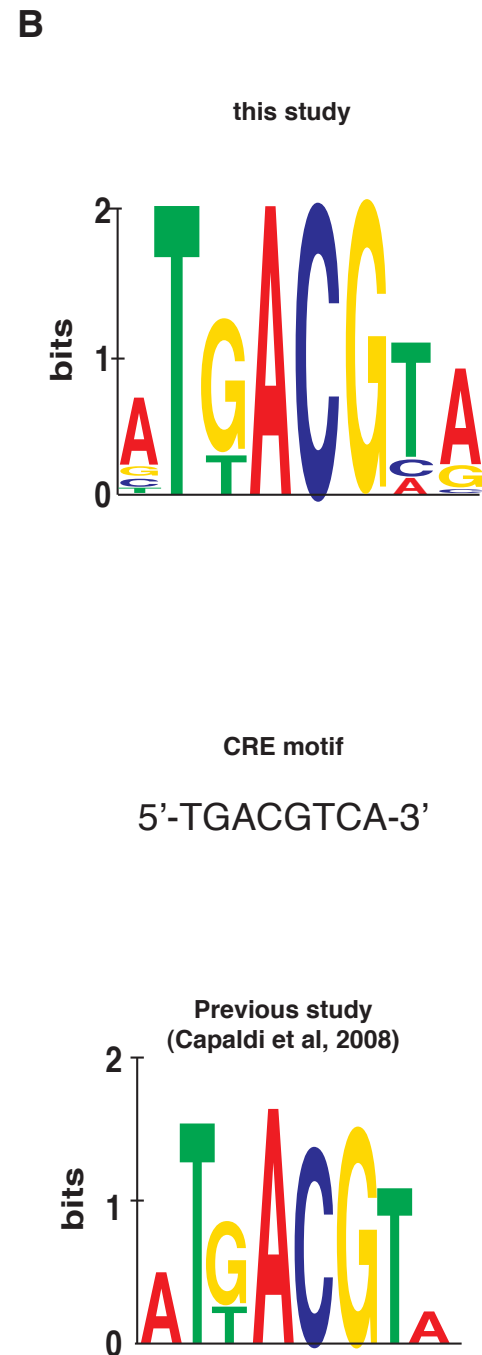
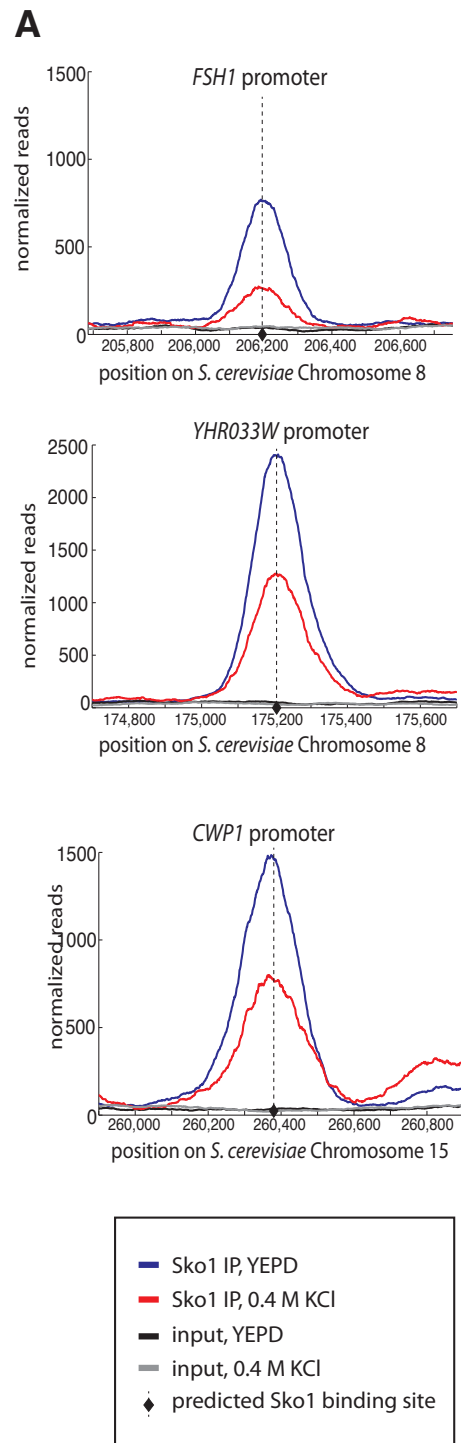
We analyzed the sequences of class I binding peaks to search for cis-elements that can explain the observed pattern of binding. First, we asked if previous observations (Harbison et al., 2004; Capaldi et al., 2008; Fordyce et al., 2010) of Sko1 binding affinity could explain the binding pattern we see at class I peaks. Of the 43 class I binding peaks that occur within defined yeast promoters, 24 contain a perfect match to a known Sko1 binding motif, ACGTCA (Harbison et al., 2004) within 150 base pairs of the center of the peak. In a search of these same promoters with a more extended Sko1 motif, defined by a position weight matrix (PWM) with consensus sequence TACGTCAT, 24 of the 43 promoters searched contain a match (p -value < 0.05). When these two tests are combined, binding can be explained by the known consensus site for 74% of the peaks (32 of the 43). Class I binding peaks are well aligned with the Sko1 consensus site, which is generally within ten base pairs of the center position of each binding peak (Figure 2.2A). In addition, we were able to discover the known Sko1 binding motif *de novo* by searching the 50 base pair sequence regions at the center of each binding peak for overrepresented sequences (Figure 2.2B).

Class I binding peaks occur at the canonical Sko1 binding motif, and binding at these sites appears to be responsible for the repressive activity of Sko1 in log growth in rich medium. Of the 15 genes that are repressed two-fold or more by Sko1 during log growth in rich medium (Capaldi et al., 2008), 12 contain class I binding peaks. (At two of the remaining promoters, we do not observe Sko1 binding in either condition, and one promoter contains a large region of sequence that is not uniquely alignable, so we cannot determine whether Sko1 is bound at this gene.)

The majority of Sko1 binding peaks observed under control conditions are class I peaks; binding at these sites is reduced to about 70% of pre-stress levels after five minutes in osmotic stress. ChIP-seq measures a population average of Sko1 binding at a particular time point, and does not account for population heterogeneity. However, the previous work suggests that the observed decrease in Sko1 binding could signify a population-wide shift in the binding equilibrium, due to physical changes that accompany osmotic shock. During the initial stage of recovery from the shock phase, transcription factor binding globally decreases for a few minutes (Proft and Struhl, 2004). This general decrease in transcription factor association is thought to be caused by an increase in the ionic strength of the nucleus, due to transient contraction of the nuclear volume. This effect is quickly relieved via the action Hog1-regulated ion channels. Previous work (Proft and Struhl, 2004) indicates that Sko1 binding drops to 10% of pre-stress levels in the first minute after osmotic shock (of a similar severity to the conditions of our experiment), and by five minutes, Sko1 binding has recovered to 50% to 80% of

Figure 2.2. Class I binding peaks are well explained by the Sko1 consensus site. **(A)** Sko1 binding peaks are centered around predicted binding sites. Sko1 binding is shown in control conditions (log growth in YEPD; blue line) and in osmotic shock (treatment 0.4 M KCl for five minutes; red lines). Corresponding input material is shown in black (control) and red (osmotic shock). A black diamond on the x-axis shows the location of a predicted Sko1 binding site based on sequence, and dashed lines project this position. **(B)** An informatic search of class I binding sequences reproduces previously reported Sko1 binding motifs. A high significance ($P < 0.01$) result of *de novo* motif discovery performed with MEME software (Bailey and Elkan, 1994) is very similar to both the CRE binding motif (recognized by all CREB family proteins), and to a previously reported motif that is overrepresented in promoters that are bound and regulated by Sko1 (Capaldi et al., 2008).

Figure 2.2, continued



pre-stress levels, depending on which promoter is assayed. Other transcription factors that are not involved in the stress response behave similarly (Proft and Struhl, 2004). The reduction in enrichment in class I binding upon stress can be explained by this previously described global, transient effect, and may not require any specific signal other than the physical changes that accompany osmotic shock.

Class II Sko1 binding behavior is not predicted by the Sko1 consensus binding site

At most of class II binding sites that were analyzed (nine out of thirteen) using a permissive threshold of p -value < 0.1 , no close match to the Sko1 consensus binding site occurs within 150 base pairs of the center of the binding peak. At one gene, *HSP12*, the promoter contains a high scoring match for the Sko1 consensus binding site, but the observed class II binding peak is centered 200 base pairs away at a location that lacks a predicted binding site, and is occupied only in stress. The predicted site overlaps with a well-positioned nucleosome, which may explain the lack of Sko1 binding observed in either condition in our study (Albert et al., 2007).

At the four class II binding peaks where Sko1 is bound in the absence of stress, but binding is enhanced upon stress treatment (as shown in Figure 2.1B for *STL1*), the location of the binding site is predicted by the Sko1 consensus site. However, since at most Sko1 consensus binding sites, Sko1 enrichment decreases upon stress (as discussed for class I peaks), the presence of a consensus binding site is not predictive of the enhanced Sko1 enrichment upon stress that characterizes class II binding behavior.

Class II binding requires Hog1, but not Hog1 phosphorylation of Sko1

Hog1 both co-localizes with Sko1 (Proft and Struhl, 2002; Capaldi et al., 2008) at target promoters and phosphorylates Sko1 (Proft et al., 2001), altering its regulatory activity. We wondered if recruitment of Sko1 to class II binding sites requires phosphorylation by Hog1. To determine the Hog1 dependence of stress induced binding at class II Sko1 binding sites, we measured Sko1 binding at a class II site in the *RTC3* promoter in the presence and absence of Hog1, as well as with a mutant copy of Sko1 that cannot be phosphorylated by Hog1. In this mutant, the three Hog1 phosphorylation sites (serine 108, threonine 113, and serine 126, identified by Proft et al., 2001) are mutated to alanine. We find that Sko1 recruitment to the *RTC3* promoter does not require Hog1 phosphorylation of Sko1 (Figure 2.3). In addition, in a microarray comparing wild-type and *sko1-S108A*, *T113A*, *S126A* cells showed no significant change in gene expression in stress at class II genes, although we did observe the previously reported effects of the Hog1 phosphorylation sites on Sko1 repression targets under control conditions (Proft et al., 2001). These genes are additionally repressed in the *sko1-S108A*, *T113A*, *S126A* strain (data not shown). The regulatory role of Hog1 phosphorylation of Sko1 seems to be limited to relieving repression, and does not control Sko1 recruitment to promoters in stress. However Sko1 recruitment to the *RTC3* promoter depends on Hog1, as Sko1 recruitment does not occur in a Δ *hog1* strain. This requirement for Hog1 may be due to a physical interaction with Hog1 that occurs at promoters, or it may be a more general effect due to other functions of Hog1 in the stress response.

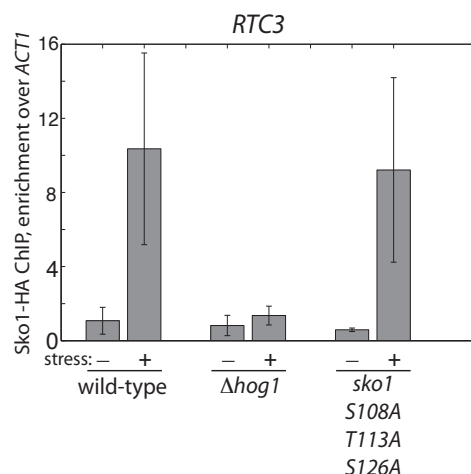


Figure 2.3. Class II binding requires Hog1, but not Hog1 phosphorylation of Sko1. Sko1 binding was measured by ChIP against the HA epitope in wild-type, $\Delta hog1$, and *sko1*- *S108A*, *T113A*, *S126A* strains, all tagged with three copies of the HA tag at the N' of the endogenous copy of Sko1. ChIP experiments were performed on cells in log growth in rich medium, in the presence or absence of osmotic shock (induced by five minutes in 0.4 M KCl.) The data points on the bar graph are the averages of three biological replicates, and error bars represent the standard deviation of the measurements. Similar results were observed at two other class II binding sites, in the *HOR2* and *STL1* promoters. We also tested a mutant version of Sko1 with PKA phosphorylation sites ablated, and found no effect on binding of expression.

Hot1 and Hog1 co-localize with Sko1 at Class II binding sites

We then asked if our list of 13 highly enriched class II Sko1 binding peaks (from the top 100 Sko1 binding peaks identified genome-wide; listed in Table 2.S1) shows significant overlap with the target gene sets of other regulatory factors that

are active during osmotic shock. We compared our peak list to published sets (Capaldi et al., 2008) of regulatory targets of the general stress transcription factors Msn2 and Msn4, regulatory targets of Hot1, promoters bound by Hot1, and promoters occupied by Hog1. We found that most promoters that show class II binding are occupied by Hog1 (9 of 13), and also bound (11 of 13) and/or regulated (8 of 13) by Hot1. To determine whether Hot1, Hog1 and Sko1 actually co-localize at promoters, or bind at distinct locations in these shared targets, we generated high resolution maps of HA-tagged Hot1 and Hog1 genome association by ChIP-seq in osmotic shock and control conditions. We find that Hot1 binding peaks occur very close to class II Sko1 binding sites (Figure 2.4A, top two panels), with maximum positions of Hot1 and Sko1 binding peaks occurring within 10 - 50 base pairs of each other. In contrast, Hot1 is absent from class I binding sites (Figure 2.4B, top two panels).

The current model explaining Hog1 presence at promoters is that both Hot1 and Sko1 are capable of recruiting Hog1 to their respective binding sites. Hot1 requires stoichiometric amounts of Hog1 to bind to promoters and activate genes (Alepuz et al., 2001; Alepuz et al., 2003), and Sko1 is also reported to induce the physical association of Hog1 with promoters (Proft and Struhl, 2002). In this model,

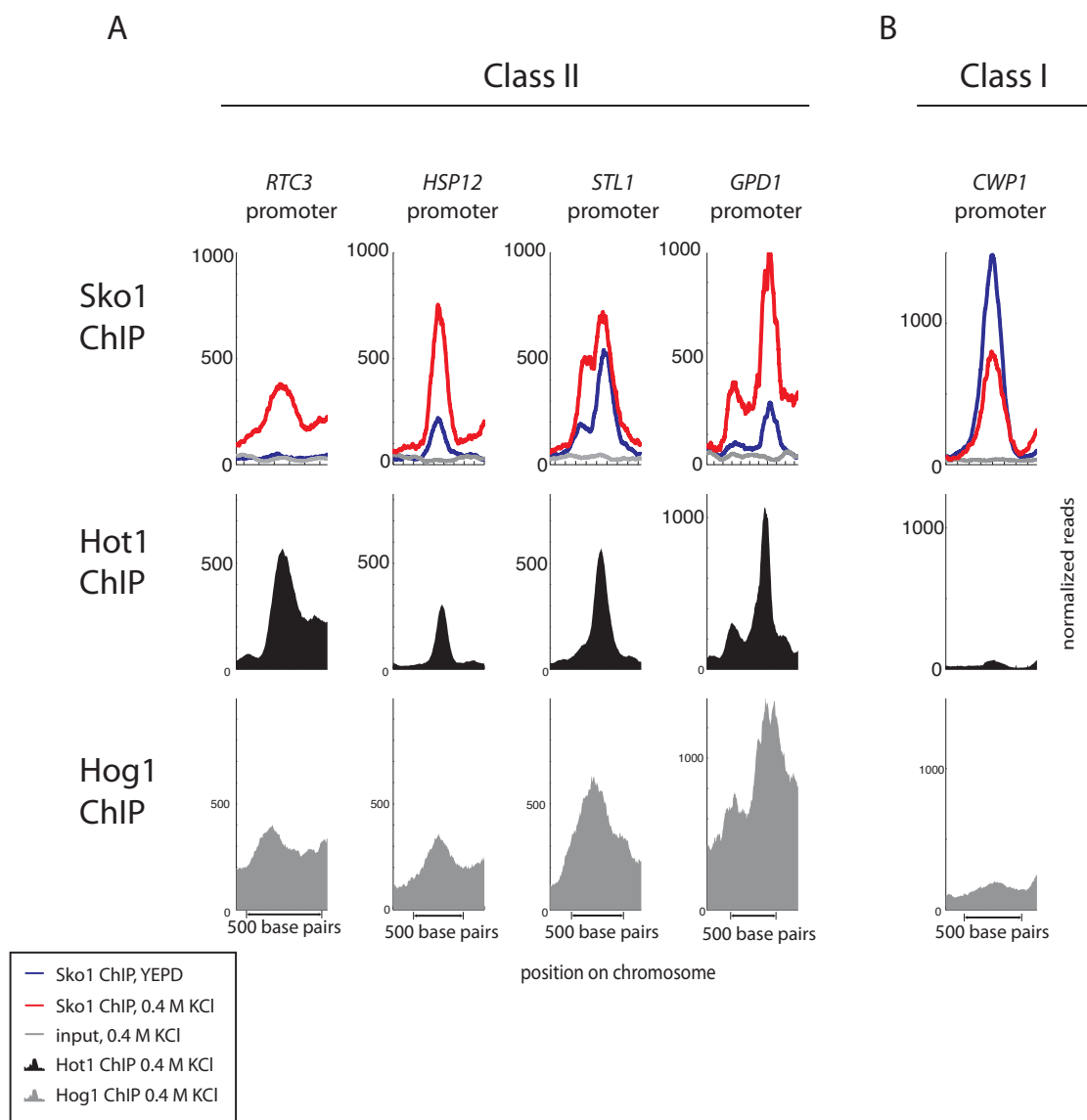


Figure 2.4 Class II binding peaks overlap with Hot1 and Hog1 promoter binding. Binding of HA-tagged Hog1 and HA-tagged Hot were measured in control conditions (log growth YEPD), during osmotic shock (5 minutes in 0.4M KCl) **A**. Sko1, Hot1 and Hog1 binding are shown for example class II peaks. Sko1 binding peaks are shown in the top panel (red, blue and gray lines correspond to YEPD IP, osmotic stress IP, and osmotic stress input sample). Hot1 binding is shown in the center panel (black filled peaks), and Hog1 binding in the bottom panel (gray filled peaks) . **B**. Sko1 binding pattern compared to Hog1 and Hot1 binding at class I Sko1 binding sites (lines and colors as in part **A**).

Sko1 and Hot1 make direct contacts with DNA, and Hog1 associates with DNA only through its interaction with these two transcription factors. Consistent with this model, the peaks we observe in Hog1 ChIP data are broader than those observed for the two transcription factors Sko1 and Hot1 (Figure 2.4A). We find that the presence of Hog1 is characteristic of class II Sko1 binding, in that Hog1 promoter peaks overlap with all sites of Sko1 recruitment in stress. While Hog1 co-localizes with Sko1 at class II binding sites (Figure 2.4,A bottom panel), Sko1 binding alone is not sufficient to recruit Hog1 to promoters. Hog1 is not recruited to class I binding sites, even those with high enrichment for Sko1 binding in stress (Figure 2.4B, *CWP1*, bottom panel).

The presence of Hog1 at class II peaks can be explained by presence of Hot1 at nearby locations. Considering previous evidence that Hot1 recruits Hog1 to promoters (Alepez et al., 2001; Alepez et al., 2003), combined with our observation that Sko1 binding alone is not sufficient to recruit Hog1 to promoters, it is likely that Hot1 is required for targeting Hog1 to specific promoters. However, Hot1 alone may not be sufficient to recruit Hog1. We observe Hot1 binding, and Hog1 promoter association, exclusively in promoters that display class II Sko1 binding behavior. At these genes, high resolution binding data suggests that Sko1 and Hot1 binding events occur in close proximity. Based on our ChIP-seq data, class II Sko1 binding, Hot1 binding, and Hog1 presence at the promoter appear to be related behaviors. (However, ChIP-seq is a population-wide measurement, so it is formally possible that Sko1, Hot1 and Hog1 do not co-localize at the same genomic locations within

individual cells.) To learn more about the sequence determinants, we searched the center regions of our observed Sko1 and Hot1 binding peaks for candidate binding motifs.

Identification of candidate binding motifs for Sko1 and Hot1

We conducted a search for overrepresented sequence motifs in class II Sko1 binding peaks, and were able to discover a somewhat poorly defined variant of the CRE binding element (Figure 2.5A, top panel). A match to this motif can be found in all class II binding peaks, with p -value < 0.005 . This motif is only well defined at four positions, and contains about nine bits of information. If this motif is in fact the cis-element recognized by Sko1 at class II peaks, additional information is likely required to specify these binding targets. Given that Hot1 and Hog1 also associate with promoters near class II binding sites, but are absent from class I Sko1 binding sites, the presence of these factors may be involved in specifying class II binding targets. We then searched the sequences of Hot1 binding peaks for overrepresented sequences. The highest significance motif discovered (Figure 2.5B, top panel) is similar to the only previously reported Hot1 candidate motif (bottom panel, reported by Capaldi et al., 2008), which was identified based on lower resolution genome-wide binding data, as well as genetic dependence on Hot1. Our candidate motif appears in all 11 of our Hot1 binding peaks, with a p -value < 0.0001 .

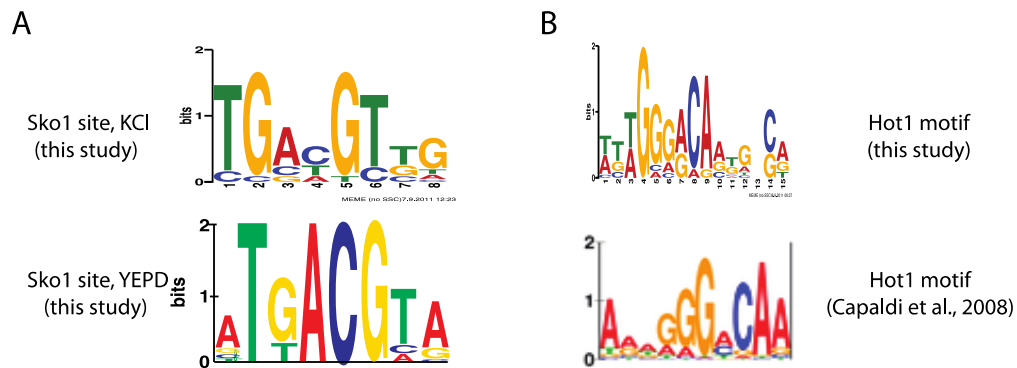


Figure 2.5 Identification of Sko1 and Hot1 binding motifs at class II promoters. We used web-hosted MEME software to conduct a search for overrepresented sequence motifs at the center (50 base pairs) of Sko1 and Hot1 binding peaks, with the constraint that we expect to find exactly one occurrence of the motif within peak. **(A)** Candidate motif for stress-only Sko1 binding. To increase our pool of sequences, we included four significant binding peaks that were not within the top 100 most highly enriched peaks, bringing the total to 17. The candidate Sko1 motif (top panel) occurs in all 17 sequences at a p-value threshold of 0.005. This motif appears to be a less constrained version of the Sko1 consensus site (bottom panel). **(B)** A search of 11 Hot1 binding peaks allows *de novo* discovery of a candidate motif for Hot1 (top panel) that is similar to the only previously reported Hot1 candidate binding motif (bottom panel).

Candidate binding motifs predict Sko1 and Hot1 binding in close proximity

We then asked if the Sko1 and Hot1 candidate motifs can explain the observed Sko1 and Hot1 binding patterns. Matches to the candidate motifs appear near the center of Sko1 and Hot1 binding peaks (Figure 2.6). In addition, the observed offset between the centers of nearby Sko1 and Hot1 binding peaks is predicted by the spacing of the Sko1 and Hot1 candidate binding motifs in the promoter. The closest spacing of Sko1 and Hot1 motifs is 10 base pairs, or about one helical turn of DNA.

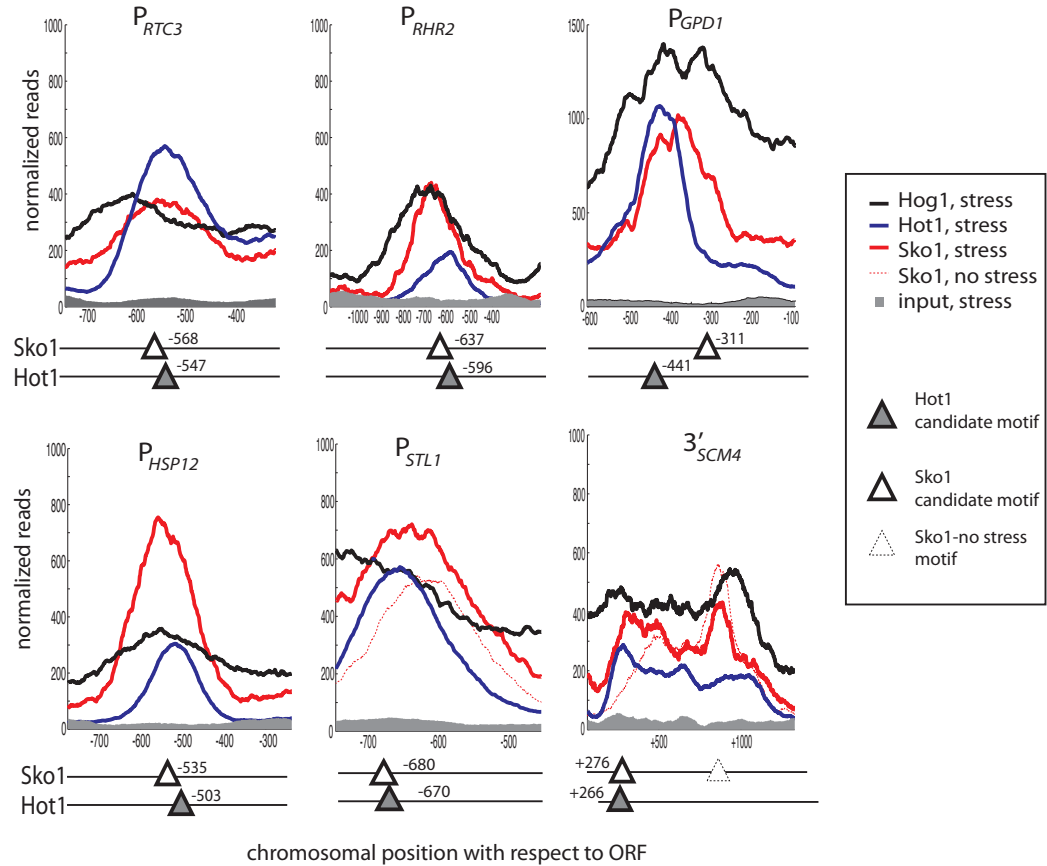


Figure 2.6. Candidate Sko1 and Hot1 binding sites appear in closely spaced pairs. Sko1 (red), Hot1 (blue) and Hog1 (black) ChIP-seq traces are shown for six class II binding peaks. These peaks are found in the regulatory regions of stress induced genes (in promoters, between 250 and 650 upstream of the transcription start site, and in one case, immediately downstream of *SCM4*.) Matches to the candidate Hot1 motif (gray triangles) and candidate Sko1 motif (white triangles) are well aligned with binding peaks. In some cases, predicted Sko1 class II binding motifs appear close to a Sko1 class I site (dashed white triangle). At these locations, both the class I and class II binding patterns are observed.

This suggests that if Sko1 and Hot1 are binding together in a complex, they each make contact with DNA. Both Sko1 and Hot1 have conserved DNA binding domains. Sko1 has a basic leucine zipper binding domain (Nehlin et al., 1992; Vincent and Struhl, 1992), and binds DNA as a homodimer both *in vivo* and *in vitro* (Pascual-Ahuir et al., 2001), at least under conditions of rapid growth. Less is known about the structure and DNA binding properties of Hot1. However, based on sequence analysis, Hot1 contains a GCR1 superfamily DNA binding domain near the C-terminus (Altschul et al., 1990).

Discussion

The results presented in this chapter demonstrate that the CREB family transcriptional regulator Sko1 has two separately regulated binding modes. In the absence of stress, Sko1 is bound to promoters at class I binding sites defined by the CRE binding motif (Figure 2.7A, left panel). Many of these promoters are repressed by Sko1 during rapid growth in rich medium. Early in the osmotic stress response, Sko1 binding to these sites decreases (Figure 2.7A, right panel). This decrease in binding, combined with the stress-specific signal of Hog1 phosphorylation, inhibits the repressive activity of Sko1 at class I binding sites, but does not activate genes to a level beyond relief of repression. Our candidate motif for class I binding is similar to Sko1 binding motifs determined by *in vivo* (Harbison et al., 2004; Capaldi et al., 2008) and *in vitro* (Nehlin et al., 1992; Fordyce et al., 2010) binding data. The decreased binding at Class I sites in stress is likely due to a global decrease in association between DNA and DNA binding proteins in the first few minutes of stress. The stress-induced decrease in Sko1 binding at class I sites is similar to the behavior of other transcription factors, both general and specific, under the same conditions (Proft and Struhl, 2004). Overall, at class I sites, Sko1 behaves canonically, recognizing a previously identified, well defined binding site, and experiencing a general decrease in binding site occupancy in the early stages of the osmotic stress response. In contrast, class II Sko1 binding is an unexpected behavior. Upon stress, Sko1 is depleted from class I binding sites and

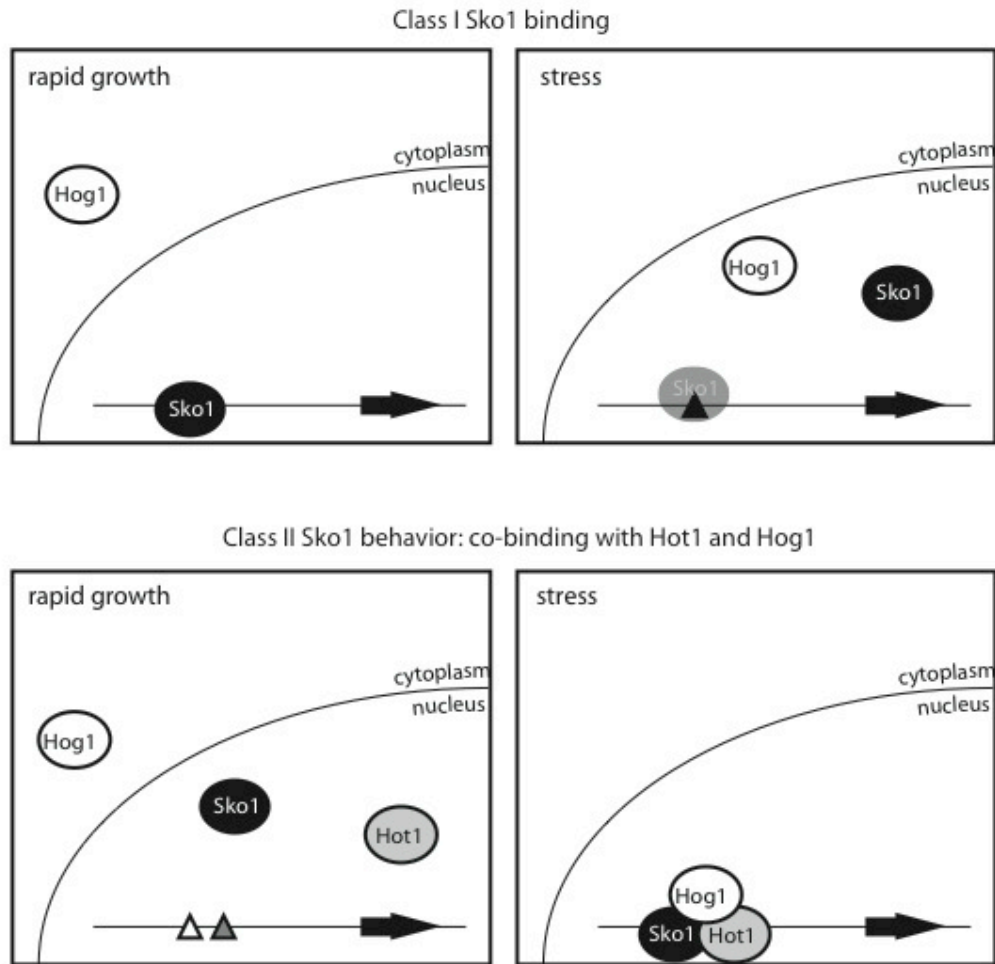


Figure 2.7. Model of two types of Sko1 binding behavior. **A.** Sko1 occupies class I binding sites in the absence of stress. At class I binding sites, Sko1 recognizes the CRE binding motif in the absence of stress. Upon stress, Sko1 dissociates from class I binding sites, due to the physical effects of stress. Hog1 is translocated into the nucleus during stress, but does not associate with Sko1 at class I sites. **B.** Class II binding sites are bound by Sko1 in response to osmotic shock. In the absence of stress, adjacent, cryptic binding sites for Sko1 (white triangles) and a co-binding factor Hot1 (gray triangles) are unoccupied. Upon osmotic shock, Hog1 localizes to the nucleus, where it binds with Hot1. A putative interaction between Sko1 and the Hot1/Hog1 complex allows Sko1 to recognize its cryptic site, and stabilizes the binding of the three proteins.

simultaneously recruited to class II sites, which contain only a weak match to the consensus CRE site. Somehow, Sko1 is able overcome a global effect that decreases transcription factor binding to occupy seemingly inferior class II binding sites. The presence of a weak CRE binding site alone is not sufficient to define genomic locations of class II binding sites, or to distinguish class II sites from class I sites. We find that at class II binding sites, Sko1 co-localizes with both the MAP kinase Hog1, which coordinates the stress response, as well as Hot1, a transcription factor that requires Hog1 as a binding co-factor. (Figure 2.7B). We identified a sequence element that is overrepresented in Hot1 binding peaks that is similar to a previous prediction of the Hot1 motif, based on binding and expression data. We find high significance matches to this putative Hot1 motif adjacent to the class II Sko1 motif, often with spacing of about 10 base pairs.

Hog1 co-localizes with Sko1 only at class II binding sites, and not at class I binding sites (where Sko1 binding can still be observed in stress, although with decreased enrichment from pre-stress levels.) This observation suggests that Sko1 alone is not capable of recruiting Hog1 to promoters, in contrast to previous models of Hog1 promoter recruitment (Proft and Struhl, 2002). Instead, we observe most Hog1 promoter association at sites where both Sko1 and Hot1 are also present. We suspect that Sko1, Hot1, and Hog1 bind as a complex, and each member of this complex may depend upon the others for binding. Further work will be necessary to determine the strength and nature of this dependence.

The two observed binding modes of Sko1 offer a model of catalytic versus stoichiometric regulation by Hog1. At class I binding sites, Sko1 repression is regulated by Hog1 phosphorylation. These genes are repressed by Sko1 under normal growth conditions. In response to stress, Hog1 phosphorylates Sko1, and these genes are derepressed. Sko1 repression is influenced by very low levels of nuclear Hog1: some inhibition of Sko1 repression due to Hog1 phosphorylation is observed even under optimal growth conditions. In addition, full relief of Sko1 repression does not require high levels of nuclear Hog1.

In contrast, class II binding, or more simply, Sko1 recruitment in stress, requires stoichiometric amounts of Hog1, while phosphorylation of Sko1 by Hog1 does not substantially affect Sko1 binding or activity at class II sites (Figure 2.3). Interestingly, under conditions of cell wall damage, when nuclear Hog1 levels remain low, Sko1 repression is completely relieved in a Hog1-dependent manner, but Sko1 activation does not occur (discussed in Chapter 4 of this work, Figure 4.4). The two observed binding modes of Sko1 suggest a mechanism by which different sets of promoters respond to increased nuclear Hog1 at different threshold.

Materials and Methods

Strains

All strains used in this study are in the W303 strain background (*trp1 leu2 ura3 his3 can1 GAL+ psi+*), and are listed in Table 3.1. Gene deletions were introduced by transformation with PCR products including auxotrophic markers flanked by the 40 bp sequence found directly upstream and downstream from the gene, followed by selection on the appropriate media (Rothstein, 1991, Brachman et al., 1998). Tagged strains (HA) were constructed in a similar way (Longtine et al., 1998) but the primers directed the recombination to the sites directly upstream and directly downstream from the appropriate stop codon. Insertion of the HA tag into the correct locus was confirmed by PCR and western blot. (HA-Sko1 was a gift from Kevin Struhl's lab.) Strains with multiple gene manipulations were constructed by mating the single delete strains and dissecting the resulting tetrads.

A Quickchange kit (Stratagene) was used to introduce amino acid substitutions to generate non-phosphorylatable Sko1 variants. First 3HA-Sko1 was cloned out of genomic DNA into YCp50, where mutations were introduced. Mutant Sko1 was reintroduced in the endogenous locus first by *URA3* replacement of Sko1 at the desired locus, followed by replacement of the *URA3* marker with mutant Sko1.

Strain	Genotype	Background
EY0690	w303 MATa	EY0690
<i>hog1</i> Δ	<i>hog1::Ura3</i> MATa	EY0690
3HA-Sko1	3HA-Sko1 MATa	EY0690
3HA-Sko1 <i>hog1</i> Δ	3HA-Sko1 <i>hog1::Ura3</i> MATa	EY0690
3HA-Sko1 S108A, T113A, S126A	3HA-Sko1 S108A, T113A, S126A MATa	EY0690
Hot1-3HA	Hot1-3HA (His3)MATa	EY0690
Hog1-3HA	Hog1-3HA (His3)MATa	EY0690

Table 2.1. Strains used in this study

Chromatin Immunoprecipitation

ChIP-seq and ChIP-qPCR experiments were conducted as described previously (Johnson et al., 2007; Robertson et al., 2007; Lee et al, 2006, Capaldi et al., 2008.) Cells were diluted from an overnight culture to an OD₆₀₀ of 0.1. Cells were grown at 30° C in YEPD, with shaking, to OD₆₀₀ 0.6. Cultures were split for stress treatment and mock treatment. For stress treatment, YEPD supplemented with KCl was added to ~120 OD units of culture, bringing the final concentration of KCl to 0.4 M; for mock treated cells, the same volume of YEPD was added to cultures. After five minutes in stress, samples were crosslinked with 1% formaldehyde at room temperature for 15 minutes. Crosslinking was quenched with 125 mM glycine for five minutes, and then samples were harvested by centrifugation, washed twice in cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) and then snap-frozen in liquid nitrogen. Samples were resuspended in 1 mL lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% Na Deoxycholate) in the presence of

protease inhibitors (Roche; Complete) and mechanically lysed by bead beading. Lysates were sonicated (9 cycles of 15 seconds each, power 2 on Misonix 3000) to solubilize chromatin and then clarified by centrifugation. Ten percent of the clarified lysate was reserved to serve as an input control. Clarified lysates (~10 mg for ChIP-seq, ~ 2.5 mg for ChIP qPCR) were incubated with 12Ca5 anti-HA antibody, for 2 hours at 4° C before addition of Protein G Dynabeads (Invitrogen). After incubation for 4 hours up to overnight, beads were washed twice with lysis buffer, once with high salt buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% Na Deoxycholate), once with lysis buffer and once with TE (10 mM Tris, 1 mM EDTA, pH 8). All washes were performed at room temperature for five minutes each, on an end-over-end mixer. Samples were eluted from beads in TE plus 0.67% SDS at 65° C for 30 minutes. Supernatants were removed from beads and incubated overnight at 65° C to break crosslinks. DNA was isolated by digestion of RNA with RNase A for 2 hours at 37° C, protein digestion with Proteinase K for 2 hours at 55° C, and purified by phenol chloroform extraction and precipitation with ethanol and NaCl. DNA pellets were stored in TE.

For ChIP-seq, ~ 10 ng IP material was used to generate each library, following the Illumina protocol for their paired end DNA sample prep kit (v1). After addition of adaptors DNA in a size range of 175-300 base pairs was isolated by gel electrophoresis for amplification. Size ranges of prepared libraries were measures on an Agilent Biol analyzer (average size 225 base pairs) before sequencing on Illumina Genome Analyzer II (performed by Christian Daly at the FAS Center for Systems Biology Core Facility). Thirty-six base reads were obtained, and aligned to

the *Saccharomyces cerevisiae* genome using ELAND (Jianwen Zhang performed the alignments). For Hog1 and transcription factor ChIP-seq samples, five million to ten million reads were obtained. Uniquely alignable sequence tags were mapped to the genome and extended by the average length of the library (minus the adaptor length) in MATLAB. For ChIP-qPCR, samples were analyzed on an MX300p qPCR machine (Stratagene) using primers that amplify a ~ 100 base pair region surrounding the center of observed binding peaks.

The HA-tagged Hot1 strain has an expression defect at some genes, so the set of Hot1 binding sites obtained from ChIP-seq of Hot1-HA may not be complete, if the tag interferes with binding at some promoters more than others.

Sequencing data analysis

Peak identification

Initial identification of peaks was performed using an implementation of the PeakSeq method (Rowosky et al.) written by Xu Zhou (described in Zhou and O'Shea, 2011). This list of peaks was screened by additional criteria: for one hundred consecutive bases, peaks must be two input standard deviations about the input value at that location, and above the overall median input value. This screened list of peaks was then sorted by enrichment over input, and the one hundred highest enrichment peaks were selected for analysis. For this purpose, enrichment was defined as the ratio of IP reads to input reads, with reads summed

over the 50 base pairs at the center of each binding peak. Complete lists of all peaks found using PeakSeq are listed in Tables S2.2 (Sko1 ChIP-seq) and S2.3 (Hot1 ChIP-seq).

Identification of class I and class II binding peaks

Peaks were classified as class I if they: 1) show binding in control (no stress) conditions; and 2) this binding is reduced in stress. Peaks are classified as class II if their enrichment value is higher in stress than in control conditions. Class II peaks may show significant enrichment in control conditions if enrichment is increased upon stress, although most do not. Of the top 100 peaks that were identified and classified, the median class I peak showed a 60% decrease in binding upon stress, with a standard deviation of 18%. For motif analysis, only those peaks from the top 100 most enriched that fall into a strictly defined yeast promoter were considered (56/100 peaks met this criteria; 43 from class I and 13 from class II). If multiple peaks in the same promoter were separately identified, both were included in the analysis (eg at *HOR7* and *STL1*).

Promoter scan with Sko1 position weight matrix

Scans of promoter regions for high scoring matches to a position weight matrix were performed using the TestMOTIF software program (Barash et al, 2005), using motifs and parameters as described by Tsankov et al., (2010). Promoters were defined as 600 base pairs upstream of each open reading frame. (Binding

peaks more than 600 base pairs away from a transcription start site were excluded from this analysis.) Jay Konieczka provided advice on the use of this software.

Identification of Sko1 and Hot1 candidate motifs

We conducted a bioinformatic search of the genomic regions enriched in each ChIP-seq experiment using the web-hosted MEME software suite. We selected the 50 bp regions surrounding the maximum height position of each peak for analysis. During motif discovery, we specified the parameter that each sequence should contain one occurrence of the putative binding motif. We scanned peaks that are bound by Hot1 in stress, bound by Sko1 with higher enrichment prestress (class I) and bound by Sko1 with higher enrichment in stress (class II). For discovery of the class II Sko1 binding motif, class I peaks were used as a set of counterexamples in a discriminatory search.

Microarrays

Measurement of gene expression changes by microarray were conducted as described previously (Capaldi et al., 2008; Zhou and O'Shea, 2011). To harvest samples for microarrays, cells were grown at 30° C in YEPD, with shaking, to OD₆₀₀ 0.6. Samples were harvested by cold methanol quenching before and after addition of 0.4 M KCl to the growth medium. Cultures were added directly to ~50° C methanol and quenched for 20 minutes, and then harvested by centrifugation. RNA was isolated using the RNase mini kit (Qiagen). cDNA was synthesized by reverse transcription using random 10-mer and poly-dT primers using Superscript (Invitrogen) , and a 2:3 ratio of amino allyl-UTP:dTTP was used to incorporate

chemically reactive groups into the cDNA. Samples were labeled with NHS-ester cy 3 or cy 5 (GE Biosciences). 300 ng of cDNA for each channel was loaded onto an 8 by 15k *S. cerevisiae* two-color expression microarray from Agilent (G2509F). Arrays were hybridized for 17 hours at 60° C and washed using Agilent buffers.

Microarrays were scanned on an Axon 4000B scanner, using GenePix 5.1 software to acquire images and extract fluorescence intensity values for each spot. Lowess normalization was performed using the MATLAB bioinformatics toolbox (version 2009a).

List of Supplemental data

Table 2.S1 List of top 100 highest enrichment binding peaks. Enrichment was calculated (as described in the methods) for each Sko1 binding peak, and the peaks with the 100 highest enrichment values in either condition were selected for farther analysis.

Table 2.S2 List of all significant peaks in Sko1 ChIP-seq data

Table 2.S3 List of all significant peaks in Hot1 ChIP-seq data

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Chapter 3

Genome-wide transcriptional reprogramming in response to osmotic shock

Introduction

Transcriptional stress responses are often described as gene expression modules (e.g. Wu et al., 2004), with the implication that these modules can be added on to an existing transcriptional program to adjust to a change in conditions. This framework is most appropriate when considering a specific problem that can be addressed by altering the transcription of a small set of genes, e.g. starvation for a particular amino acid. However, transcriptional responses to environmental stresses, such as osmotic shock, heat shock, or oxidative damage, affect hundreds of genes, increasing their expression levels by up to two orders of magnitude in within minutes or tens of minutes (Gasch et al., 2000). In addition, these responses rapidly coordinate the production of hundreds of copies of specific mRNAs. This means that the transcriptional rate at the most highly induced genes in stress may exceed the maximum rate of transcription observed for any gene under conditions of rapid growth (about 4 mRNAs per minute, Miller et al 2011).

Large scale responses are more akin to a temporary reprogramming of transcription than a modular addition. If cells in log growth are operating close to their transcriptional capacity, it may not be possible to add the stress response to the existing transcriptional program. Early in the stress response, cells may need to divert some transcriptional resources away from the rapid growth transcription program to allow rapid induction of stress responsive genes.

S. cerevisiae responds to increased osmolarity with rapid induction of hundreds of genes (Gasch et al., 2000; Capaldi et al., 2008). This transcriptional

response occurs within minutes of stress, with many genes reaching maximal expression levels by 10 minutes, and some target genes are induced over 100 fold above their pre-stress levels. A stress response of this scale puts considerable demands on the cell's transcriptional resources. The transcriptional response to osmotic stress is coordinated by the MAP kinase Hog1 (Brewster et al., 1993). Upon stress, Hog1 is localized to the nucleus (Ferrigno et al., 1998), where it regulates the action of several transcription factors (Rep et al., 2000, Proft et al., 2001; Proft and Struhl, 2002 ; Alepuz et al., 2001). In addition, Hog1 forms a complex with RNA Pol II that is present during elongation at a subset of the stress responsive genes (Pokholok et al., 2006; Proft et al., 2006).

Hog1 may also play a more general role in allocation of transcriptional resources during the early phase of the stress response. In the first few minutes following osmotic shock, transcription rates globally decrease (Proft and Struhl, 2004; Romero-Santacreu et al., 2009; Millet et al., 2011). Specifically, transcription initiation rates drop, apparently due to decreased association of general and specific transcription factors with genomic DNA factors (Proft and Struhl, 2004). This effect is partially attributed to the effects of increased ionic strength of the nucleoplasm, due to a contraction of volume. These physical effects inhibit transcription initiation in the first few minutes of stress, although elongating polymerase complexes are not affected, and transcription factor binding resumes by five minutes after stress treatment. However, about 50% of this global decrease in transcription rate depends on Hog1 (Romero-Santacreu et al., 2009), suggesting that

the physical effects of stress are not the sole cause of the observed global decrease in transcription.

In this study, we investigate the role of Hog1 in allocating transcriptional resources in the early stages of stress. We find that a RNA Pol II-Hog1 complex transcribes most highly expressed genes during stress. This complex is preferentially directed to a set of stress responsive genes that are bound and regulated by the Hog1 cognate transcription factors Sko1 and Hot1. The location of candidate Sko1 and Hot1 binding sites provide a possible explanation for previously implicated downstream sequence elements in the regulation of these genes.

Results

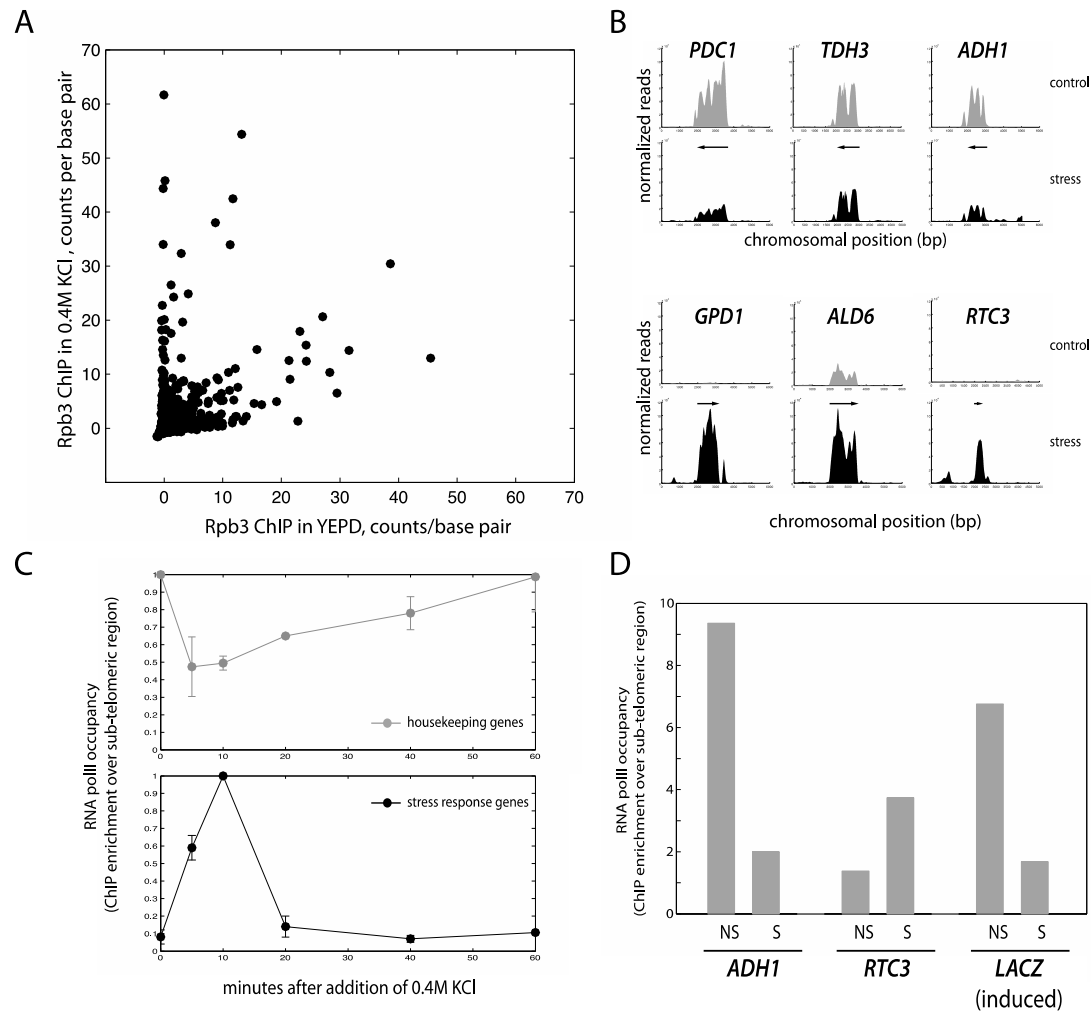
Global reallocation of Pol II during osmotic shock

To investigate allocation of transcriptional resources during the early stages of the *S. cerevisiae* osmotic stress response, we measured RNA polymerase location genomewide by ChIP-seq of Rpb3, the third largest subunit of RNA Pol II. In order to observe the transition from the initial shock phase of the stress response to the induction phase, we selected an early time point (5 minutes after addition of 0.4 M KCl) to compare to mock-treated cells. After five minutes in stress, most genes are depleted of Pol II, while a smaller set of genes gains Pol II (Figure 3.1A). (These results are in general agreement with work from earlier this year (Miller et al., 2011) that includes a similar experiment, conducted at a later time point in under harsher osmotic shock conditions.)

Before stress treatment, cells deploy the highest levels of RNA Pol II to genes that encode the highly abundant metabolic enzymes involved in glycolysis and fermentation (Figure 3.1B, top panel, gray plots). During recovery from stress, RNA Pol II is depleted from these genes (Figure 3.1B, top panel, black plots), and stress-specific genes are briefly the most highly transcribed genes in the genome (Figure 3.1B, bottom panel, black plots), despite very low-level expression before stress (Figure 3.1B, bottom panel, gray plots). This switch in polymerase occupancy occurs rapidly, but is short-lived. By five minutes, stress-induced genes are near their peak Pol II occupancy levels, and within twenty minutes, Pol II occupancy has

Figure 3.1 A switch in RNA Pol II occupancy upon stress. **(A)** In response to stress, RNA Pol II, is depleted from most genes, but recruited to smaller group of stress-induced genes. RNA Pol II occupancy was measured by ChIP-sequencing of Rpb3, the third largest subunit of RNA Pol II, in control (mock-treated with YEPD) cells, and cells subjected to five minutes of osmotic shock, induced by addition of 0.4M KCl to the growth medium. The scatter plot compares RNA Pol II occupancy in unstressed cells (x axis) to stressed cells (y axis). ChIP-sequencing data for the two conditions is normalized to the same total number of sequencing reads. Each point on the plot represents one gene. Coordinates for each gene are determined by summing the total number of reads that align within the open reading frame for ChIP and input samples, subtracting the input signal from the ChIP signal, and normalizing by the length of the gene. Data plotted are from one sequencing run per sample. The same trend is apparent in a lower quality sequencing dataset (not shown) and has been confirmed by QPCR at several genes. **(B)** The set of genes with highest RNA Pol II occupancy switches upon stress. Genes with the highest levels of RNA Pol II present in the ORF before stress experience a loss of polymerase upon stress (top panel, highest ranking genes in control conditions are shown. Compare pre-stress RNA Pol II occupancy in gray to stress occupancy, in black). Upon stress, a different set of genes shows the highest RNA Pol II occupancy (bottom panel, highest ranking genes after 5 minutes in stress). Many genes with high RNA Pol II occupancy in stress were not expressed at a high level in control conditions. Data plotted are from one sequencing run per sample. The same trend is apparent in a lower quality sequencing dataset (not shown) **(C)** Both depletion and recruitment of RNA Pol II are rapid, transient effects. Dynamics of depletion and recruitment of RNA Pol II in stress were measured by a timecourse of ChIP-QPCR experiments.. Cells in log growth were subjected to osmotic shock, as described in **A**. Genes with high RNA Pol II occupancy pre-stress are quickly depleted of Pol II upon stress, and begin recovering within 10 minutes (gray, average behavior of *PDC1*, *TDH3*, *ILV5*). Stress induced genes recruit polymerase upon stress treatment and then rapidly return to low RNA Pol II occupancy. (black line, average behavior of *RTC3* and *STL1*.) Data shown are the average of two experiments. **(D)** RNA Pol II is depleted from an exogenous expression system upon stress. The TET-ON system was used to drive expression of P_{TET07} -*LACZ*, encoded on a plasmid, to a high level. Induced cells were then subjected to osmotic shock (as described in **A**) and RNA Pol II occupancy was measured at *LACZ*, as well as a control genes that show high Pol II occupancy pre-stress (*ADH1*) or during stress (*RTC3*).

Figure 3.1, continued



fallen substantially at these genes (Figure 3.1C, black line). Pol II occupancy at metabolic enzyme genes follows an inverse pattern, dropping by five minutes in stress, and then recovering (Figure 3.1C, gray line).

Depletion of RNA Pol II upon stress is the default gene behavior

Why are high expression genes depleted of RNA Pol II upon stress, while stress genes are induced to a very high level? Perhaps depletion is a gene specific effect. Alternatively, depletion of RNA Pol II may be a general, global phenomenon, due to either to the physical effects of stress or some trans-acting signal. To differentiate between these two classes of explanations, we drove the expression of an exogenous, plasmid encoded gene (*LACZ*) using the Tet-On induction system. We then measured RNA Pol II occupancy in induced cells in the presence or absence of osmotic stress (five minutes in 0.4 M KCl) at *LACZ* and at two control genes. As expected, RNA Pol II is depleted from the highly expressed metabolic enzyme *ADH1* upon stress, and recruited to the stress-induced gene *RTC3*. Induced *LACZ* behaves like the *ADH1* control: *LACZ* is highly expressed before stress, and RNA Pol II is depleted from *LACZ* upon stress. This result demonstrates that depletion of RNA Pol II is the default outcome upon stress, rather than a gene-specific effect.

During the early stages of response to osmotic shock, RNA Pol II is depleted from genes that were previously highly transcribed, while stress induced genes gain RNA Pol II. This switch in polymerase occupancy occurs very quickly, and soon thereafter, both classes of genes return to their pre-stress expression levels. Depletion of RNA Pol II from highly expressed genes does not seem to rely on

specific cis-encoded elements, given that an exogenously expressed gene driven by an exogenous expression system demonstrates the same Pol II depletion effect as endogenous high expression genes. If depletion of RNA Pol II upon stress is the default behavior, then stress-activated genes, especially those with a very high absolute level of expression, must be regulated by a mechanism that overrides this effect.

Hog1 occupies highly transcribed open reading frames in stress

Although stress-activated factors suffer the same dissociation effects as other transcription factors (Proft and Struhl, 2004), stress induced genes achieve maximal transcriptional rates before most genes have recovered to their pre-stress transcription levels (Romero-Santacreu, 2009, Miller et al., 2011). We were interested in how induction of the stress genes occurs simultaneously with a genome-wide decrease in transcription. Given that the stress-induced genes are the exceptions to the global, general effect of RNA Pol II depletions, we are interested in general differences between transcription at stress induced genes *versus* Pol II-depleted genes in during stress.

One mechanism of interest is the association of Hog1 with elongating RNA Pol II during stress. Under osmotic shock conditions, the MAP kinase Hog1 forms a complex with RNA Pol II and is present in some ORFs during elongation (Proft et al., 2006). At these genes, Hog1 is part of the elongating RNA Pol II holoenzyme complex. Hog1 has been observed in the ORFs of several stress-induced genes during osmotic shock (Proft et al., 2006; Pokholok et al., 2006). Of the

approximately 275 Hog1-dependent genes expressed in stress (Capaldi et al., 2008), only 45 are reported to be occupied by Hog1 (Pokholok et al., 2006). Interestingly, these 45 genes are among the most highly occupied by RNA Pol II in stress in our data. To investigate relationship between high RNA Pol II occupancy and the presence of Hog1 in ORFs during stress, we measured Hog1 occupancy of ORFs in the presence and absence of stress (0.4 M KCl for 5 minutes) by ChIP-seq against Hog1-HA, and then compared these measurements to those obtained for Rpb3. We find that in stress, Hog1 occupies the ORFs of nearly all highly transcribed genes. Hog1 presence in the open reading frame is roughly proportional to RNA Pol II occupancy (Figure 3.2A, top panel), consistent with previous reports that Hog1 is part of the elongating RNA Pol II holoenzyme (Proft et al., 2006). This relationship between gene expression and Hog1 presence during elongation is specific to stress, and does not occur in control conditions (Figure 3.2A, bottom panel).

These trends hold true when we examine the highest expression genes in both stress and control conditions. In stress, Hog1 is present in the both the promoters and coding regions of highly transcribed genes (Figure 3.2B). The presence of Hog1 in promoters during stress is expected, based on previous measurements (Alepuz et al., 2001; Alepuz et al., 2003; Proft and Struhl, 2002; Capaldi et al., 2008). In the absence of stress, when Hog1 is mostly absent from the nucleus, Hog1 is not observed in highly transcribed ORFs, and is absent from promoters as well (Figure 3.2C).

By comparing Hog1 presence in the ORF to RNA Pol II occupancy, we are able to see relationships between Hog1 occupancy and absolute expression levels during stress. This relationship is difficult to see in comparisons between Hog1 ORF occupancy and fold change in gene expression (e.g. from microarray studies), since genes with very high fold change may never be very highly expressed in absolute terms. This situation is common, as many stress-induced transcripts are barely detectable in the absence of stress (Miller et al., 2011). Based on the additional information gained by considering expression levels of Hog1 occupied ORFs, we find that in stress, Hog1 is present with RNA Pol II at nearly all highly transcribed ORFs, rather than just a small subset. Given the high rate of transcription occurring at these genes, the physical association between Hog1 and elongating Pol II may affect a larger fraction of RNA Pol II than previously thought. Further work will be necessary to determine how much RNA Pol II associates with Hog1 during stress, and whether this complex exists free of DNA or assembles during initiation.

Hog1 cognate transcription factors enhance the presence of Hog1 in ORFs

Although Hog1 lacks a DNA binding domain, it is able to associate with promoters through physical interactions with its cognate transcription factors Sko1 (Proft and Struhl, 2002) and Hot1 (Alepuz et al., 2001; Alepuz et al., 2003). In addition to forming a stable complex with transcription factors, Hog1 also interacts directly with the major subunit of RNA Pol II. This interaction has been observed with purified proteins *in vitro*, and the polymerase co-immunoprecipitates with

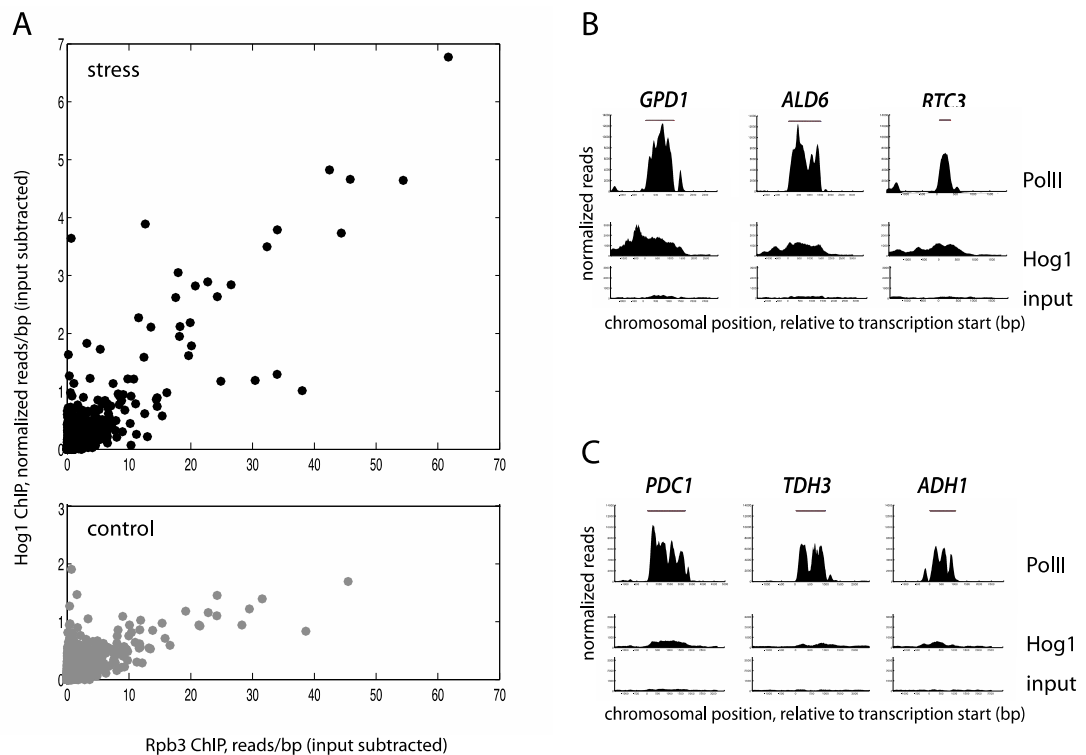


Figure 3.2. Hog1 occupies the ORFs of the most highly transcribed genes during stress. **(A)** Upon treatment with 0.4M KCl, Hog1 occupies open reading frames in proportion to Pol II occupancy. Scatter plots show ChIP-seq of Hog1-HA compared to ChIP-seq of Rpb3, after 5 minutes of osmotic shock with 0.4M KCl (top panel), and in control, mock-treated cells (bottom panel). ChIP-sequencing data for all samples is normalized to the same total number of sequencing reads. Each point on the plot represents one gene. Coordinates for each gene are determined by summing the total number of reads that align within the open reading frame, and normalizing by the length of the gene. **(B)** Hog1 occupies the promoters and ORFs of the highest expression genes in stress. Plots show Rpb3 ChIP, Hog1-HA ChIP, and sequenced input for the three highest expression genes in stress, ranked by polymerase occupancy (those at the top of the plot in Figure 3.1A). **(C)** Hog1 occupancy of highly transcribed ORFs is specific to stress. Plots show Rpb3 ChIP, Hog1-HA ChIP, and sequenced input for the three highest expression genes in control conditions (log growth in YEPD), ranked by polymerase occupancy (those at the right of the plot in Figure 3.1A). The data used to generate these plots is available in Table S2.1.

Hog1 under osmotic stress conditions (Proft et al., 2006). Taken together, these observations suggest that Hog1 may have a function analogous to that of Mediator complex, acting as a go-between for RNA Pol II and transcriptional activators that bind directly to DNA.

The current model for recruitment of the Hog1/ RNA Pol II complex to ORFs is that 3' downstream regions identify target genes (Proft et al., 2006), although no specific cis regulatory element has been identified. The Hog1-regulated transcriptional response to osmotic shock involves the transcription factors Sko1 and Hot1, which are known to physically associate with Hog1 at promoters, as well general stress transcription factors Msn2 and Msn4. Msn2 and Msn4 exert at least partial control over the majority of stress induced genes, while the role of Sko1 and Hot1 is more limited (Capaldi et al., 2008). Although Sko1 and Hot1 target a smaller set of genes, those target genes tend show the highest levels of RNA Pol II and Hog1 occupancy during stress. Because Sko1 and Hot1 are known to recruit Hog1 to promoters, it seems likely that these factors could be involved in the recruitment or assembly of the Hog1/RNA Pol II complex. We measured Sko1 and Hot1 binding to promoters by ChIP-seq (see Chapter 2 for full discussion of these ChIP-seq experiments) and compared the binding of these factors to occupancy of ORFs by Hog1 and RNA Pol II. We observe that most highly expressed genes exhibit a specific pattern of binding behavior. These genes' promoters, and in some cases, immediate 3' downstream regions, contain cryptic Sko1 and Hot1 binding sites, located in close proximity. At these promoters, an apparent complex of Sko1, Hot1, and Hog1 is recruited in stress by the class II binding mechanism described in

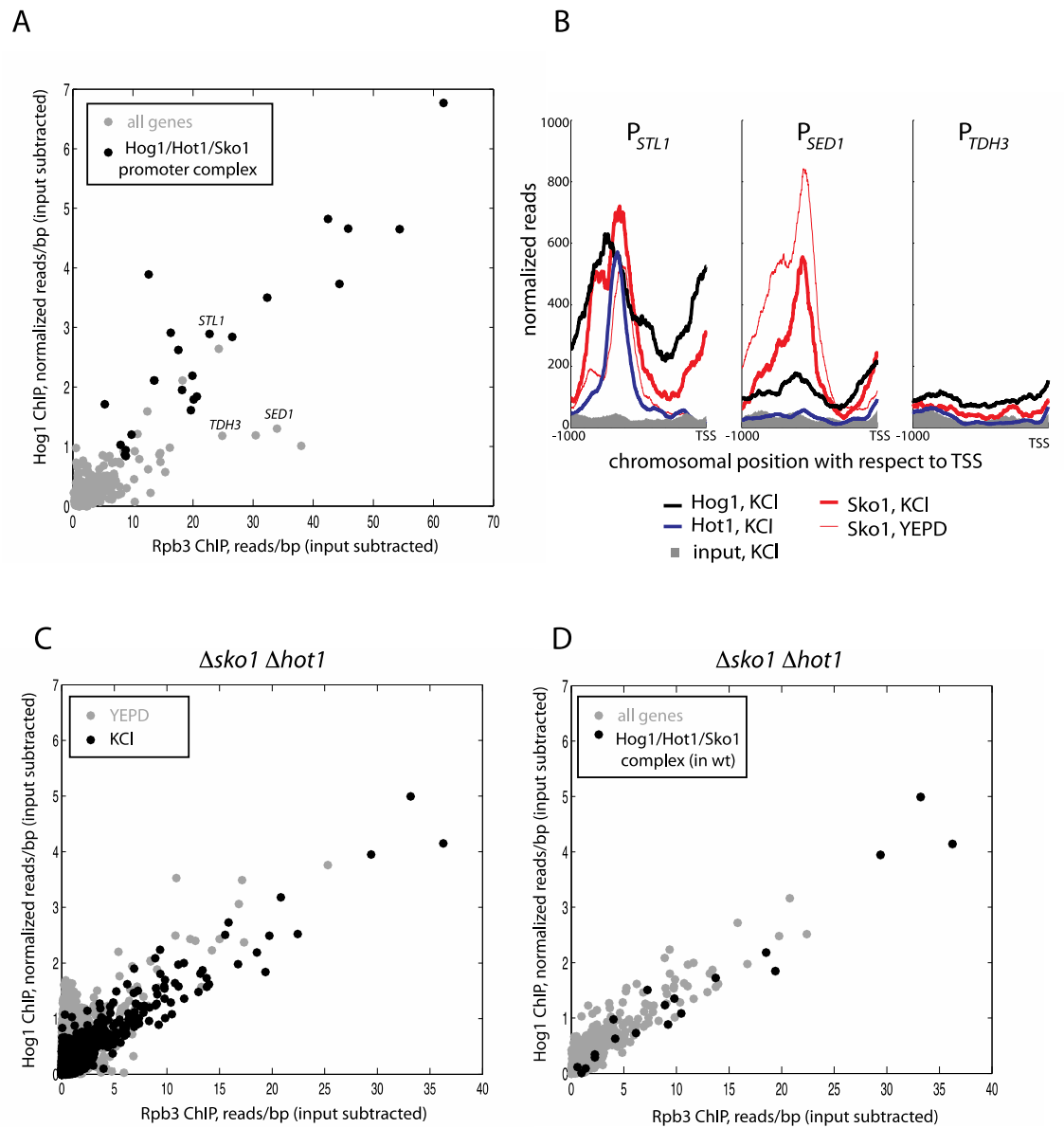
Chapter 2. (Figure 3.3A; these genes are listed in Table 3.1). In addition, genes bound by Sko1, Hot1 and Hog1 show enhanced recruitment of Hog1 in the ORF, compared to other genes with a similar level of RNA Pol II occupancy (Figure 3.3B). This comparison is possible only for moderately expressed genes; genes with the highest Pol II occupancy are uniformly Sko1 or Hot1 bound. This trend suggests Hog1/ RNA Pol II complexes preferentially occupy genes that are marked by Sko1, Hot1 and Hog1 at regulatory regions.

ORF	Gene name
YDL022W	GPD1
YDL222C	FMP45
YDR536W	STL1
YER062C	HOR2
YFL014W	HSP12
YGL037C	PNC1
YGR049W	SCM4
YGR052W	FMP48
YGR086C	PIL1
YGR088C	CTT1
YGR243W	FMP43
YHR087W	RTC3
YHR094C	HXT1
YIL053W	RHR2
YJL107C	YJL107C
YJL108C	PRM10
YMR251W-A	HOR7
YOL151W	GRE2
YOR317W	FAA1
YOR348C	PUT4
YPL061W	ALD6
YPR149W	NCE102

Table 3.1. List of genes that display co-binding of Sko1, Hot1 and Hog1.

Figure 3.3. A promoter-bound complex of Hog1 and its cognate TFs Sko1 and Hot1 enhances Hog1 ORF occupancy at specific genes. **(A)** In stress, genes bound by a Sko1/Hot1/Hog1 complex show highest levels of RNA Pol II and Hog1 in ORFs. Scatter plot shows ChIP-seq of Hog1-HA compared to ChIP-seq of Rpb3, after 5 minutes of osmotic shock with 0.4M KCl, as in Figure 3.2A. Genes are color coded by binding behavior of the Hog1, Sko1, and Hot1. Genes that exhibit co-localization Sko1, Hot1 and Hog1 in regulatory region are shown in black (e.g. *STL1*). Genes that display Sko1 binding alone (without Hot1 and Hog1 e.g. *SED1*), or completely lack Sko1 or Hot1 binding (e.g. *TDH3*) are shown in gray. Only genes with RNA Pol II enrichment above background (defined as two standard deviations above the median input level) are shown (677 genes total). Sko1 and Hot1 binding behavior was determined by ChIP-sequencing; see Methods. Example genes shown in **B** are labeled on the plot. **(B)** Example genes of the classes compared in **A**. These three example genes are occupied by RNA Pol II at similar levels, but recruit different amounts of Hog1 to the ORF. P_{STL1} is bound by Sko1 in the absence of stress (dashed red line), but Sko1 binding increases in stress (solid red line) when Hot1 (blue line) and Hog1 (black line) bind at a nearby genomic location. In contrast, P_{SED1} shows a decrease in Sko1 enrichment upon stress, and is not bound by Hot1 or Hog1. P_{TDH3} is not bound by Sko1, Hot1 or Hog1 in either condition. **(C)** Hog1 recruitment to ORFs is absent in a strain lacking Sko1 and Hot1. Scatter plot shows ChIP-seq of Hog1-HA compared to ChIP-seq of Rpb3, in control conditions (gray dots) and after 5 minutes of osmotic shock (black dots) with 0.4M KCl in a $\Delta hot1 \Delta sko1$ strain. **(D)** Promoters that recruit the Sko1/Hot1/Hog1 complex to promoters in wt cells no longer show enrichment of Hog1 in ORFs in a $\Delta hot1 \Delta sko1$ strain. Genes shown are those expressed above background in wild-type cells (as in **A**), with the same color-coding.

Figure 3.3 continued



In a strain lacking Sko1 and Hot1, this set of genes no longer shows enhanced recruitment of Hog1 to ORFs (Figure 3.3B). Many of these Sko1 and Hot1-dependent genes are still induced in response to stress in $\Delta hot1 \Delta sko1$ cells through redundant mechanisms of activation, relying on the general stress factors Msn2 and Msn4, although their expression levels are decreased (Capaldi et al 2008). In $\Delta hot1 \Delta sko1$ cells, there is no increase in Hog1 occupancy in highly expressed ORFs upon stress (Figure 3.3C). At genes that were bound by these factors in wild-type cells, an elevated Hog1/RNA Pol II ratio is no longer observed in $\Delta hot1 \Delta sko1$ cells (Figure 3.4D).

Predicted Hot1 and Sko1 co-binding sites occur in 3' regions required for Hog1 ORF recruitment

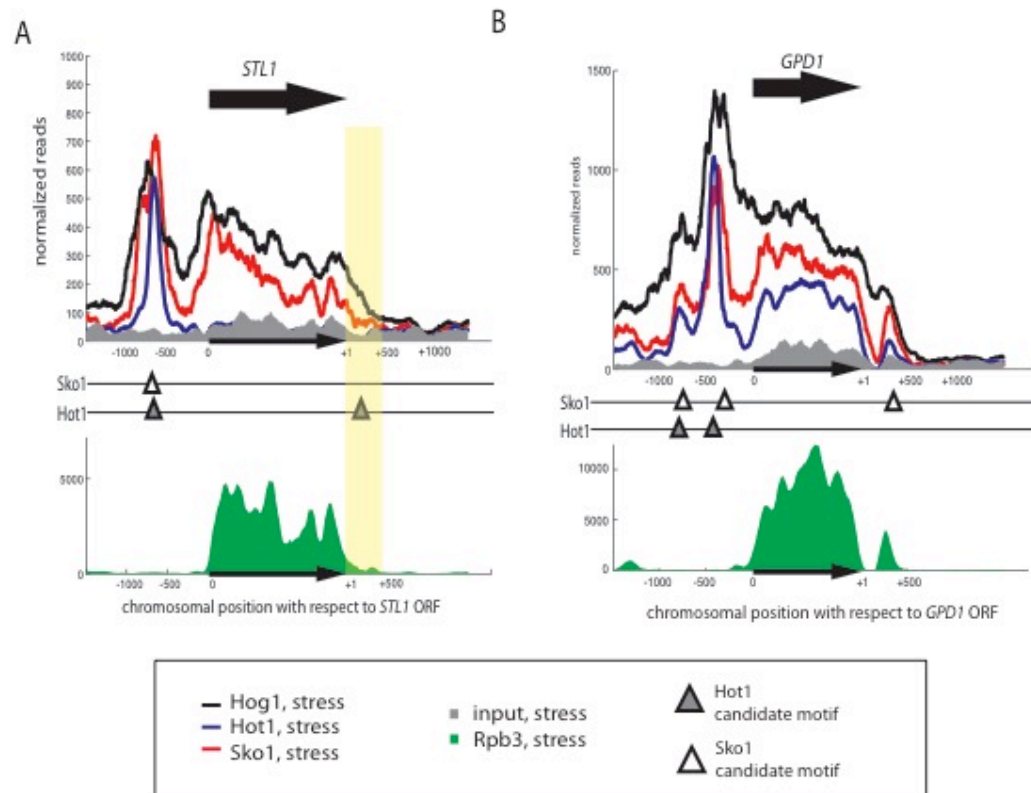
Previous reports indicate that at least some genes that recruit Hog1 to the ORF contain a necessary and sufficient cis regulatory element in the genomic region immediately downstream of the gene. (At *STL1*, the best characterized example of this behavior, this required region extends about 400 base pairs downstream of the ORF, extending farther downstream than the 3' untranslated region.) We find a predicted Hot1 binding site within this required downstream region of *STL1* (highlighted region in Figure 3.4), in addition to predicted and observed Sko1 and Hot1 binding in the promoter (Figure 3.4A). However, we do not observe Hot1 binding at this location. Our HA-tagged Hot1 has a moderate expression defect at

STL1, and binding to this site may be impaired in the tagged strain. Additional work will be necessary to determine if wild-type Hot1 binds to this site.

We do predict and observe Sko1 binding downstream of *GPD1* (Figure 3.4B), another gene that recruits Hog1 to the ORF during stress (see Figure 3.2B), and at three other genes, suggesting a role for the downstream regions in promoting transcription, perhaps through a looping mechanism (Figure 3.4C). Interestingly, the observed Sko1 binding site downstream of *GPD1* occurs overlaps with a region that is moderately enriched for TATA binding protein during stress (Pascual-Ahuir et al., 2006). Both Sko1 and Hot1 are likely to be binding to DNA as dimers; Sko1 binds as a dimer (Nehlin et al., 1992; Vincent and Struhl, 1992; Pascual-Ahuir et al., 2001) as does Gcr1, the closest homolog of Hot1 (Deminoff, S., and Santangelo, G., 2001), which may increase the number of possible interactions that can occur between these proteins and Hog1. Hog1 is the only member of this complex known to be capable of recruiting the polymerase, suggesting that the role of Sko1 and Hot1 is simply to ensure the recruitment of Hog1 to these promoters.

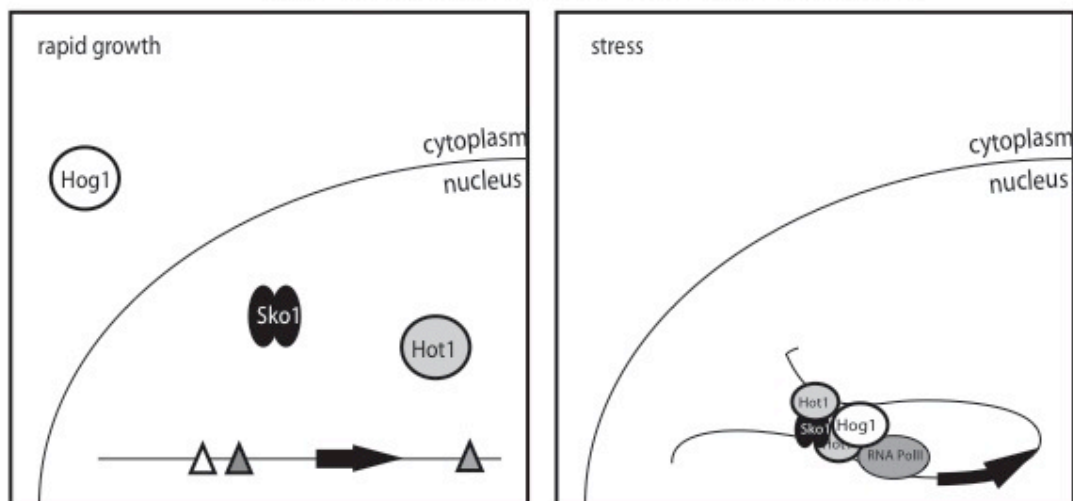
Figure 3.4. Predicted and observed Sko1 and Hot1 binding suggests a role for downstream regulatory regions. **(A)** Diagram showing predicted and observed binding of Sko1 and Hot1 in the regulatory and coding regions of *STL1*. Plot shows ChIP-seq data collected from samples taken during osmotic shock (5 minutes in 0.4M KCl) for Sko1 (red line), Hot1 (blue line), Hog1 (black line) and Rpb3 (green) compared to input DNA from the same conditions (gray). Locations of predicted stress-specific Sko1 and Hot1 binding sites (white triangles and gray triangles, respectively) are shown. Highlighted region (pale yellow) was shown previously to be necessary and sufficient for ORF occupancy of Hog1 (Proft et al., 2006). **(B)** Diagram showing predicted and observed binding of Sko1 and Hot1 in the regulatory and coding regions of *GPD1*. **(C)** Possible model for the role of downstream Sko1 and Hot1 binding sites in regulating transcription. In the absence of stress (left panel), Sko1, Hot1 and Hog1 do not occupy cryptic binding sites in the promoter or downstream region. In response to stress, Hog1 is translocated into the nucleus, prompting binding of the Sko1/Hot1/Hog1 complex. The stoichiometry of this complex is not known. The Sko1/Hot1/Hog1 complex may be stabilized by DNA looping that brings binding sites located on opposite ends of the ORF into close proximity.

Figure 3.4 continued



C

proposed model for role of downstream Sko1 and Hot1 binding sites



Discussion

In response to osmotic shock, we observe a redistribution of RNA Pol II upon stress. The polymerase is directed away from the set of genes that were previously highly expressed, while simultaneously transcribing a set of stress-induced genes at a rate that may exceed the highest rate of transcription that occurs in the absence of stress. We find that depletion of the polymerase from the ORF is the default gene behavior, suggesting that stress-responsive genes must be equipped with some kind of override that exempts them from this effect.

An RNA Pol II- Hog1 elongating complex was shown previously to form during stress, but the role of this complex in the stress response was not clear (Pokholok et al., 2006; Proft et al., 2006). We demonstrate that this complex selectively transcribes the most highly induced stress genes. Two Hog1 cognate transcription factors, Sko1 and Hot1, bind together at cis regulatory elements in the promoters, and in some cases, downstream regions of stress responsive genes, targeting the Hog1-RNA Pol II complex to these genes. In the absence of Sko1 and Hot1, no difference in Hog1 ORF occupancy is observed upon stress, suggesting that these factors are required for the recruitment, or formation, of a Hog1-RNA Pol II complex.

The RNA Pol II- Hog1 complex transcribes highly expressed, stress-induced genes, while avoiding genes that were previously highly expressed. The apparent selectivity of this complex suggests a mechanism by which the stress induced genes can be exempted from the transient global transcriptional repression that

accompanies stress. Hog1 seems to provide the override mechanism that allows stress-responsive genes to be transcribed despite global repression, but there is evidence that Hog1 plays a role in causing this repression as well. Interestingly, the stress-induced global decrease in transcriptional rate is partially dependent on Hog1 (Romero-Santacreu et al., 2009). In wild-type cells, we and others (Romero-Santacreu et al., 2009; Miller et al., 2011) observe about a 50% drop in transcription genome-wide. In the absence of Hog1, only about a 25% drop occurs (Romero-Santacreu et al., 2009) or half of the global reduction in transcription that is observed in wild-type. The stress induced global reduction of transcription may have two causes: one, a general effect of the physical changes that accompany stress, as suggested by Proft and Struhl (2004); and two, a Hog1-dependent signal that increases this effect.

What type of Hog1 dependent signal would be capable of globally altering the transcription rate? Such a signal would need to interact with the general transcriptional machinery. Given that Hog1 forms a complex with the polymerase upon stress, it is possible that the physical presence of large amounts of Hog1 in the nucleus can serve as this signal. In this model, RNA Pol II in complex with Hog1 is not capable of transcribing non-stress responsive genes, allowing Hog1 to briefly hijack copies of RNA Pol II to carry out its specified transcription program (Figure 3.5). A model in which Hog1 alters RNA Pol II selectivity via a stoichiometric interaction requires that relatively large amounts of this complex form in response to stress. While an association between Hog1 and RNA Pol II has been observed *in vivo* and *in vitro* (Proft et al., 2006), no estimates of the stoichiometry or abundance

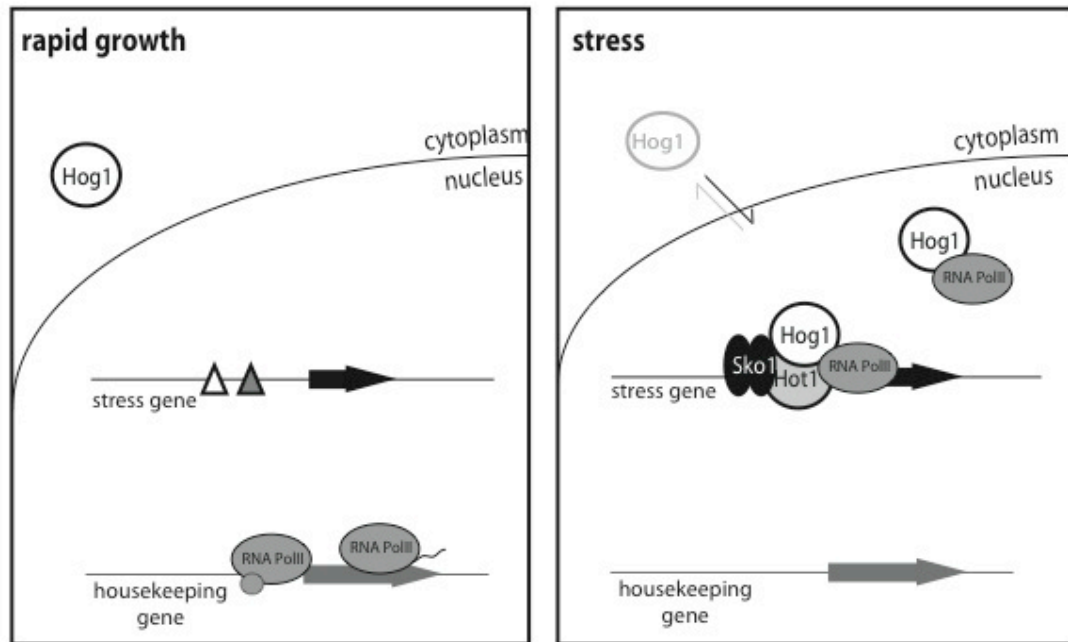


Figure 3.5. Model for Hog1 reprogramming of transcription in stress. Under conditions of rapid growth, Hog1 is localized the the cytoplasm, and a set of housekeeping genes are highly transcribed, while stress genes are transcribed at a very low rate. In response to stress, Hog1 enters the nucleus, where it forms a complex with some fraction of RNA Pol II holoenzymes. These Hog1-modified polymerases selectively transcribe genes with promoter-bound Hog1 cognate transcription factors.

of this complex have been made. It would be interesting to see if Hog1 modifies a large enough fraction of RNA Pol II to possibly account for the Hog1-dependent 25% drop in transcription upon stress observed by Romero-Santacreu and colleagues (2009).

If large amounts of nuclear Hog1 are capable of globally modifying the transcription rate, we would expect spurious activation of Hog1 to be detrimental. In fact, overexpression of Hog1 causes growth arrest, and deletions of upstream inhibitors of the Hog1 pathway are not viable. Feedback mechanisms in the Hog1

pathway (Macia et al., 2009) make it difficult to induce activation of Hog1 in the absence of stress through tricks of signaling (see Materials and Methods), although other approaches may be more effective.

The function of polymerase depletion in the early stages of the osmotic stress response may be to reallocate transcriptional resources, rather than to modify the expression level of the RNA Pol II-depleted genes. Depletion of RNA Pol II from highly expressed ORFs does not have the same biological effect as classical transcriptional repression, because many of the transcripts produced by Pol II depleted genes are in fact stabilized during the stress response (Miller et al., 2011). It is not clear if a transient global decrease in transcription is a characteristic of other environmental stress responses. However, the partial Hog1 dependence of this effect suggests that it may not simply be a physical quirk of altered osmolarity, but a functional, regulated strategy to allow the cell to rapidly switch its transcriptional program.

Materials and Methods

Strains

Most strains used in this study are in the W303 strain background (*trp1 leu2 ura3 his3 can1 GAL+ psi+*), and are listed in Table 3.1. Gene deletions and tagged strains (HA) were generated as described in the Materials and Methods section of Chapter 2 of this work. HA-Sko1 was a gift from Kevin Struhl, and the plasmids (pSH0134, pSH0195 and pSH0096) and base strain (SH003) used to test the diverter scaffold strategy for activation of Hog1 were a gift from Wendell Lim.

Strain	Genotype	Background
EY0690	w303 MATa	EY0690
<i>hog1</i> Δ	<i>hog1::Ura3</i> MATa	EY0690
3HA-Sko1	3HA-Sko1 MATa	W303
3HA-Hog1	3HA-Hog1(His3)	EY0690
3HA-Hog1 <i>sko1</i> Δ <i>hot1</i> Δ	3HA-Hog1(His3) <i>sko1::leu2 hot1::ura3</i> MATa	W303
3HA-Sko1 S108A, T113A, S126A	3HA-Sko1(His3) S108A, T113A, S126A MATa	EY0690
Hot1-3HA	Hot1-3HA(His3) MATa	EY0690
SH003	<i>ste5</i> Δ <i>pbs2</i> Δ <i>ssk2</i> Δ <i>ssk22</i> Δ MATa	SH003

Table 3.2. Strains used in this study

Chromatin immunoprecipitation

ChIP experiments were conducted as described in Chapter 2, with the following modifications. RNA Pol II was immunoprecipitated with a polyclonal antibody against Rpb3 (1Y26, from Neoclone). Wildtype Rpb3 ChIP data that appears in figures comes from one ChIP-seq experiment that produced 9×10^7 – 1×10^8 reads. The results from this experiment are consistent with the results of a lower quality, replicate data set (10^6 reads per sample). In addition, ChIP-seq results have been confirmed by qPCR at selected genes (*PDC1*, *ILV5*, *TDH3*, *ADH1*, *PMA1*, *STL1*, *RTC3*, *HSP12*).

Designation of Sko1, Hot1 and Hog1 bound genes

Due to expression defects in Hot1-HA tagged strains, data from Hot1 ChIP with two different tags was combined to determine Hot1 binding: genome localization of HA-tagged Hot1 was determined by ChIP-seq, as described in Chapter 2, and previously published ChIP-chip results for TAP-tagged Hot1 (Capaldi et al, 2008) was also used as a criteria to identify Hot1 bound regions. Promoters that display Hot1 binding in either of the two tagged strains are counted as Hot1 bound. Two genes (*SCM4* and *HOR2*) with clear binding peaks immediately downstream of the ORF, rather than upstream, are included in the Sko1 Hot1 bound group.

Inducible LACZ

YCP50 plasmid carrying P_{TET07} -*LACZ*, cloned from pCM173 was produced and transformed into a strain expressing rtTA from the *MYO2* promoter, integrated at the *URA3* locus. (Strains used for inducible expression are the same as in Kim and O'Shea, 2008.) Dox concentrations ranging from 0.1 to 2 $\mu\text{g}/\text{mL}^{-1}$ doxycycline were tested to determine an appropriate working concentration that would drive RNA Pol II occupancy of the *LACZ* construct to a level similar to that of the most highly expressed endogenous genes. Based on approximately 5 hours of growth in rich medium at 30° C after addition of doxycycline, 1.25 $\mu\text{g}/\text{mL}^{-1}$ doxycycline was determined to be an appropriate concentration of inducer.

For the experiment, cells were grown to saturation overnight, and then diluted to OD₆₀₀ 0.1. At this point, doxycycline was added to induce *LACZ* expression.

Attempts to send Hog1 into the nucleus in the absence of stress

We attempted two different, signaling-based methods of driving Hog1 into the nucleus in the absence of stress. Since Hog1 phosphorylation controls its nuclear import, we monitored Hog1 phosphorylation by western blotting with a anti phospho-P38 antibody that recognizes phosphorylated Hog1. (Cell Signaling Technologies) as a proxy for Hog1 nuclear localization and activity. We carried out pilot experiments using the diverter scaffold system engineered by Park et al., which cross wires PBS2 (the MAP kinase kinase upstream of Hog1) to the mating pathway. In the diverter scaffold strain, osmotic stress response genes can be induced in response to α -factor, although this induction occurs on a much longer time scale than the wildtype stress response. While we did observe α -factor induced osmotolerance in the diverter scaffold strain, we were not able to activate Hog1 to even 10% level achieved by osmotic stress in wildtype cells, even after several hours of exposure to 20 μM α -factor.

In addition, we tested a tet-repressible Sln1 construct. Sln1 is an upstream inhibitor of Hog1 pathway signaling, and Sln1 delete cells are not viable. We found that the

repressible Sln1 cells had slightly elevated basal Hog1 activity in the absence of inducer. Once inducer was added, the growth rate of these cells slowed substantially, although high levels of Hog1 phosphorylation were not observed. After six hours in the presence of 1.25 $\mu\text{g}/\text{mL}^{-1}$ doxycycline, growth of these cells had slowed to a doubling time of over eight hours.

Supplemental Data

Table 3.S1 ChIP-seq data used in this study.

ChIP-seq data for Rpb3, Hog1, Sko1 and Hot1, as well as sequenced input material are included in this table. These datasets were normalized by the total number of sequencing reads in the YEPD input sample. Values reported for each gene are normalized sequencing reads per base pair. To generate these values, counts for each sequencing dataset were summed over the length of each gene's ORF, and then divided by the ORF length.

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Chapter 4

Structure of the Hog1-activated transcriptional network in response to cell wall damage

Introduction

The p38 mitogen activated protein kinases (MAPKs) are found in organisms ranging from yeast to human, where they play an important role in triggering a variety of stress responses (Zarubin and Han, 2005). Studies in mammals have revealed that a single p38 kinase, p38 α , is activated in response to a wide range of stimuli, including UV light, heat, osmotic shock, oxidative damage, inflammatory cytokines and growth factors (Bulavin et al., 1999; Han et al., 1994; Harris et al., 2000; Rouse et al., 1994; Zarubin and Han, 2005). Once activated, p38 α phosphorylates and activates numerous target proteins, including the transcription factors ATF-1/2/6, Sap1, CHOP, p53, MEF2C, MEF2A, USF1, DDIT3, ELK1, NFAT and HBP1 (Galibert et al., 2001; Gomez del Arco et al., 2000; Han et al., 1997; Huang et al., 1999; Janknecht and Hunter, 1997; Pereira et al., 2004; Tan et al., 1996; Thuerlauf et al., 1998; Waas et al., 2001; Wang and Ron, 1996; Yang et al., 1999; Yang et al., 1998; Yee et al., 2004). These transcription factors then affect many cellular processes including inflammation, apoptosis, the cell cycle, development and senescence (Han et al., 1997; Haq et al., 2002; Takenaka et al., 1998; Xia et al., 1995).

The effects of p38 on transcription and cellular function appear to depend upon the stimuli acting on the cell (Ashwell, 2006; Zarubin and Han, 2005). This stress-type dependent output is likely created by integration with other signaling pathways as well as variation in the level and timing of p38 activity (Zarubin and Han, 2005). However, the mechanisms that allow p38 to trigger different outputs are poorly understood.

The p38 kinase Hog1 in *S. cerevisiae* is also thought to play a role in responding to different stress conditions (Alonso-Monge et al., 2001; Lawrence et al., 2004; Panadero et al., 2006; Singh, 2000; Winkler et al., 2002), suggesting that it might be a useful model for studying condition specific output by p38 and other MAPKs. However, previous studies of Hog1 function have focused almost exclusively on its role in high salt stress. In these conditions, at least two membrane bound sensors (Sho1 and Sln1) activate Hog1 through separate signaling pathways that converge on the MAPK kinase Pbs2 (Posas and Saito, 1997; Posas et al., 1996). Once phosphorylated by Pbs2, Hog1 is imported into the nucleus, where it activates multiple transcription factors (TFs), including Sko1, Hot1 and the partially redundant factors Msn2 and Msn4 (Msn2/4), leading to the induction of hundreds of genes (reviewed in Hohmann, 2002). Detailed microarray analysis has led to a comprehensive model of this transcriptional network and reveals that these TFs act in different modes and combinations to induce gene expression in response to KCl stress (Capaldi et al., 2008).

Here we examine the role of Hog1 in cell wall damage, and compare the data to that measured previously for osmotic shock induced by high salt and high glucose conditions. We find that Hog1 activates different gene expression programs in different stress conditions. Using the transcriptional network in KCl as a framework, we then build a detailed model explaining how stress-type changes in Hog1-dependent gene expression occur.

Results

Stress-type Dependent Gene Activation by Hog1

To examine the function of the Hog1-dependent transcriptional network, we studied gene expression in cell wall damage (CWD) and compared both the overall response and the role of Hog1 to that found previously in osmotic shock (Capaldi et al., 2008). CWD was induced by addition of the enzyme zymolyase (which cuts β -1,3 polysaccharide linkages in the cell wall) to the medium. A previous study of CWD revealed that the Sko1 binding motif is present in the promoter of many genes upregulated in this stress, suggesting a role for Hog1 (which activates Sko1) in the response (Boorsma et al., 2004). However, few of the genes activated in osmotic stress are induced in CWD (Boorsma et al., 2004) and thus the influence of Hog1 in CWD, if it exists, must be significantly different than that found in KCl. We therefore examined gene expression in CWD to see if we could detect rearrangements in the Hog1 transcriptional network.

To elucidate the role of Hog1 in the response to CWD, we measured the transcriptional response (20 min after stress; see Methods) in wild-type and *hog1 Δ* cells using DNA microarrays (in triplicate, Tables S1 and S2) and compared it to the response found previously in osmotic shock (Capaldi et al., 2008). Remarkably, these data show that Hog1 activates a different gene expression program in each of the three stress conditions examined (Figure 4.1).

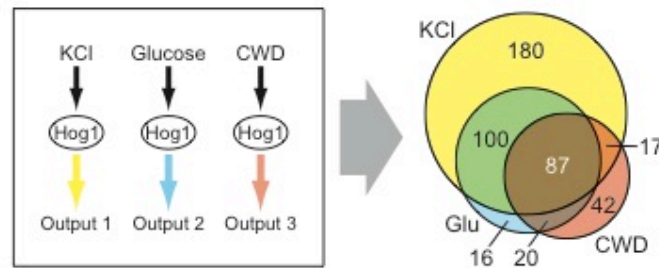


Figure 4.1. Stress-type specific gene activation by Hog1. DNA microarrays were used to determine the role of Hog1 in the transcriptional response to 0.4 M KCl, 0.8 M glucose and cell wall damage (CWD) by examining gene induction in a wild-type strain (wt stress/ wt prestress) and by comparing the expression in the wt and *hog1* Δ strains both in stress (all arrays were measured in triplicate). As shown in the Venn diagram, we found that Hog1 activates a distinct set of genes in each stress condition (Table 4.S1). Here, each circle includes all of the genes activated >1.5-fold by Hog1 ($p < 0.05$) in the stress indicated. Only genes up-regulated >1.5 fold ($p < 0.05$) in the wt strain were included in the analysis to reduce the number of false-positives included in the diagram.

Dissection of the Changes in Hog1 and Msn2/4 Activity

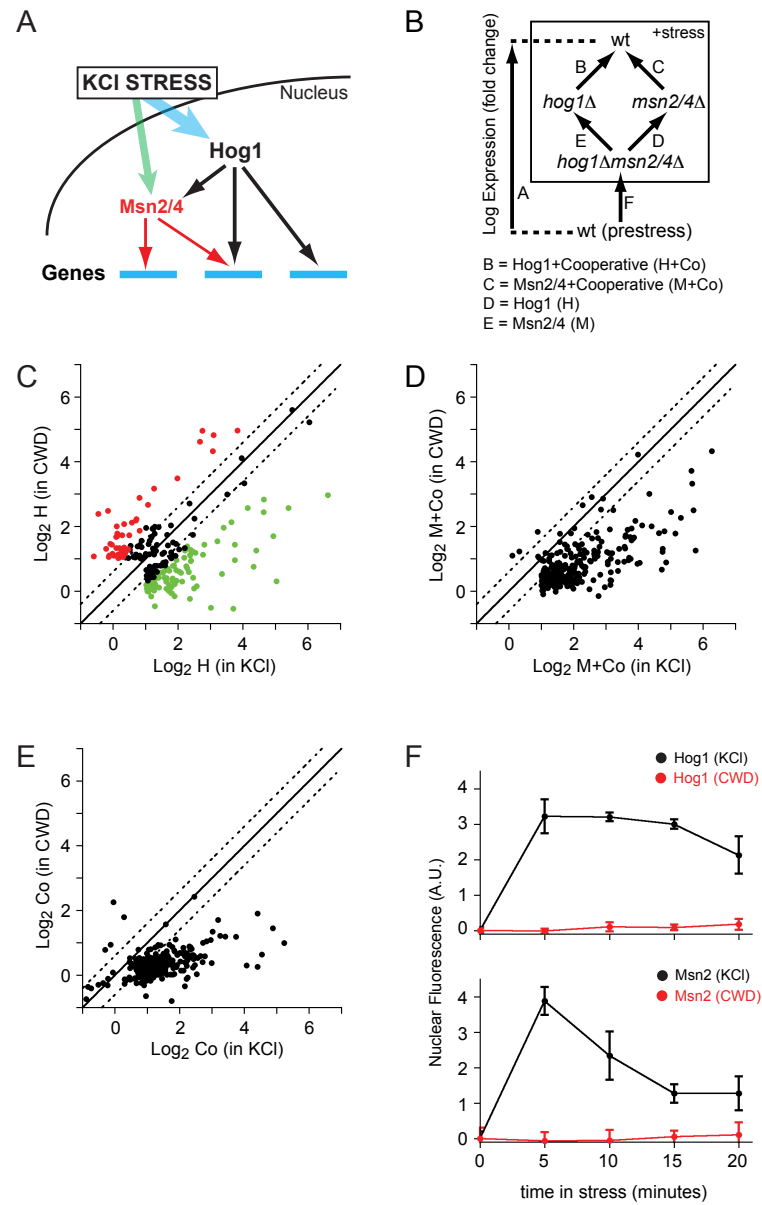
To determine how this flexible output is created, we set out to determine where in the Hog1 transcriptional network the stress-type specific changes occur. Hog1 activates genes through the general stress TFs Msn2/4, as well as through Msn2/4-independent paths (Figure 4.2a) involving activation of Sko1, Hot1 and at least one unknown transcription factor. Using a quantitative epistasis analysis approach (Figure 4.2b, described in detail in Capaldi et al., 2008), the influence of Hog1 in KCl stress can be separated into these two general mechanisms: Hog1

activation that requires the general stress TFs Msn2/4 (Co component), and Hog1 activation without Msn2/4 (H component) for each gene. To do this we directly measured the influence that Hog1 and Msn2/4 have on expression alone (*i.e.* each in the absence of the other) by comparing the expression levels of *hog1Δmsn2Δmsn4Δ* to *msn2Δmsn4Δ* and *hog1Δmsn2Δmsn4Δ* to *hog1Δ*, respectively (D and E in Figure 4.2b). These values are then compared to the influence that Hog1 and Msn2/4 have in wild-type cells (*hog1Δ* versus wild-type and *msn2Δmsn4Δ* versus wild-type; B and C in Figure 4.2b) to determine the extent of any cooperative (Co) interaction between the factors (see equations Figure 2b). Each of the four arrays (B through E in Figure 4.2b) was measured in triplicate, and the data was fit simultaneously to the equations listed in Figure 4.2b. Here, we used the same approach to dissect the influence of Hog1 in CWD (data and fitted components in Table 4.S2). We then compared the fitted expression components from these new data to those measured previously in osmotic stress.

There are dramatic differences in the influence that Hog1 has on gene expression in CWD through factors other than Msn2/4 (H component) when compared to high KCl conditions (Figure 4.2c). While many genes are less activated by the kinase in CWD (green genes, Figure 4.2c), others are more or newly activated (red genes, Figure 2c, in line with our original observations (Figure 4.1). In addition to these changes, there is a large decrease in Msn2/4 activity in CWD *versus* KCl

Figure 4.2. Stress-type dependent influence of Hog1 and Msn2/4 in KCl and glucose. **(A)** Model of the Hog1 controlled transcriptional network in 0.4 M KCl. Osmotic stress leads to the activation of both Hog1 and Msn2/4, the former through the HOG signaling pathway (blue arrow), the latter through an unknown mechanism (green arrow). Hog1 then induces gene expression both through Msn2/4 (Co component) and through a Msn2/4 independent mechanism (H component). **(B)** Schema describing the experiments and equations used to break the influence of Hog1 and Msn2/4 into components. Each arrow (labeled A-E) represents a single microarray (measured in triplicate) comparing gene expression in two strains. The equations listed below the diagram describe the relationship between the data from each measurement and the underlying expression components. **(C)** Plot comparing the influence of Hog1 without Msn2/4 (H component) on gene expression, in KCl stress (0.4 M) and CWD. Each point shows the data for a single gene; colored red if there is a significant increase ($(H_{\text{Glu}} - H_{\text{KCl}}) < 1.5$, $p < 0.05$), colored green if there is a significant decrease ($(H_{\text{KCl}} - H_{\text{Glu}}) < 1.5$, $p < 0.05$), and black if there is no significant change. The solid and broken lines show the values expected for perfect correlation and a ± 1.5 -fold difference, respectively. Data is shown for all genes with a significant H component ($H < 1.5$ -fold, $p < 0.01$) in KCl or CWD ($n = 215$). **(D)** Plot comparing the total influence of Msn2/4 (M component + Co component), in 0.4 M KCl and cell wall damage. Data is shown for all genes with a significant (M+Co) component ($(M + \text{Co}) < 1.5$ -fold, $p < 0.01$) in KCl or CWD ($n = 283$); the lines are as in C. **(E)** Plot comparing the cooperative influence of Hog1 and Msn2/4 (Co component) on gene expression, in osmotic stress due to 0.4 M KCl or CWD; the lines are as in C (the genes are those shown in D, $n = 283$). **(F)** Time-course of Hog1 and Msn2 nuclear import during KCl and CWD stress. Fluorescence microscopy was used to measure the nuclear concentration of Hog1-GFP (top panel) and of Msn2-GFP (bottom panel), in live cells, after exposure to the stress indicated in the legend. Each time-point shows the average and standard deviation from three replicate experiments. Each experiment includes data from 100-200 cells with the nucleus in focus as judged by Nhp6a-RFP signal (also used to define the nuclear boundary).

Figure 4.2 (continued)



(Figure 4.2d). Most of this change is due to a decrease in Hog1 dependent activation of Msn2/4 (Co component, Figure 4.2e). Consistent with this we do not detect significant nuclear import of Msn2 in CWD (Figure 4.2f, bottom panel).

To help determine the origin of these widespread changes in gene expression, we examined the activity/nuclear localization of Hog1 using microscopy. Surprisingly, very little Hog1 is transported into the nucleus in CWD compared to KCl (Figure 4.2f, top panel) or glucose. We found this result puzzling, as there are 47 genes induced to a significantly *higher* level by Hog1 in CWD than in KCl (red genes, Figure 4.2c). Since most of these genes are activated in CWD but not in KCl (Figure 4.2c) or glucose, one explanation for these data is that Hog1 activates a new TF in CWD. In considering this possibility, we noticed that several of the CWD-specific genes are known targets of Rlm1 (Jung and Levin, 1999), a transcription factor that plays an important role in the response to cell wall damage (Boorsma et al., 2004; Heinisch et al., 1999). In addition, another group has reported that the activity of Rlm1 is partially Hog1 dependent in a related, but much less severe, cell wall damage condition (García et al., 2009).

Activation of Rlm1 by Hog1

To test if Rlm1 is activated by Hog1 in CWD, we first analyzed gene expression in a strain with this factor deleted, both before and after treatment with KCl, glucose and CWD. We find that Rlm1 is active in CWD, but not in salt or glucose, and is important for the activation of many Hog1 dependent genes (Figure 4.3a), including

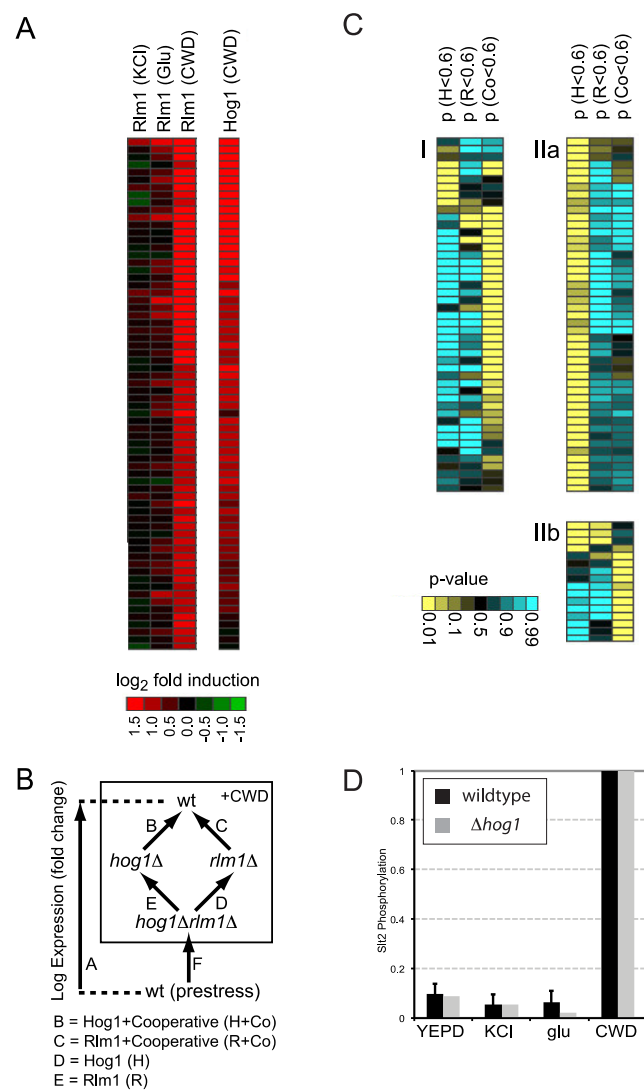
36/47 of the genes that are hyper-activated by Hog1 in CWD (red genes Figure 3a). To confirm that Hog1 is required for Rlm1 activity in CWD, and is not just acting at the same genes, we then applied the mutant cycle approach to dissect the influence and interaction of Hog1 and Rlm1 (Figure 4.3b). This analysis revealed that 87% (53/61) of genes activated by Rlm1 in CWD ($p < 0.01$) have a statistically significant ($p < 0.05$) cooperative interaction with Hog1 (Table 4.S3). Thus, Hog1 activity is required for gene induction by Rlm1 in CWD. The interaction between Hog1 and Rlm1 leads to the induction of genes involved in a wide range of process including cell wall synthesis (12/46 annotated genes) and Hog1/Slt2 pathway signaling (8/46 annotated genes).

To study the role of Hog1 and Rlm1 in more detail, we next examined the influence that Hog1 alone, Rlm1 alone and the cooperative interaction between these factors (H, R and Co components, respectively, Figure 4.3b) have on gene expression in CWD. We find that most (35/47) of the genes hyper-induced in CWD (red genes, Figure 4.2c) require an interaction between Hog1 and Rlm1 for activation and thus have a Co expression component (Group I, Figure 4.3c). By contrast, many of the genes activated to a similar or decreased level in CWD *versus* KCl (black and green genes, Figure 3a) are activated by Hog1 alone, in the absence of Rlm1 (H component, Group IIa, Figure 3c). However, there are a few exceptions (Co Component, Group IIb, Figure 3c) where Rlm1 compensates for some or all of the decrease in activation from other Hog1-dependent factors that are more active in KCl than CWD.

Together, these data show that Hog1 and Rlm1 cooperate to regulate gene expression in CWD, but not in KCl or glucose, and that this cooperation is required for Hog1 dependent induction of the genes hyper-induced in CWD (red genes, Figure 2a). While in theory the interaction between Hog1 and Rlm1 could be established through an unknown Hog1 dependent TF that is bound to almost all hyper-induced genes (many of which are only activated in CWD) and is required for Rlm1 activity, such a scenario seems unlikely. We therefore conclude that Hog1 directly, or indirectly, activates Rlm1 in CWD, but not in KCl or glucose. Together, these data show that Hog1 and Rlm1 cooperate to regulate gene expression in CWD, but not in KCl or glucose, and that this cooperation is required for Hog1-dependent induction of the genes hyper-induced in CWD (red genes, Figure 2a). While in theory the interaction between Hog1 and Rlm1 could be established through an unknown Hog1 dependent TF that is bound to almost all hyper-induced genes (many of which are only activated in CWD) and is required for Rlm1 activity, such a scenario seems highly unlikely. We therefore conclude that Hog1 directly, or indirectly, activates Rlm1 in CWD, but not in KCl or glucose.

Figure 4.3. Stress-type dependent activation of Hog1 and Rlm1. **(A)** Rlm1 activity depends on stress-type. The influence that Rlm1 has on gene expression was measured by comparing expression of *rlm1Δ* to the wt strain, both treated with stress for 20 min. The data shown is the average from at least two experiments and is corrected for the influence of Rlm1 in YEPD (which averages 1.2-fold across the genes shown here). Genes are shown in the plot if they are activated >2-fold by Rlm1 in one or more of the three stress conditions examined. The influence of Hog1 in CWD (measured by comparing expression in *hog1Δ* and wt strains) is shown for comparison. **(B)** Schema describing the experiments and equations used to break the influence of Hog1 and Rlm1 into components (as in Figure 4.2b). **(C)** Interaction between Hog1 and Rlm1. Heat map showing the statistical significance of Hog1 and Rlm1 expression components for the genes in Figure 2c. Group I shows the expression components for genes that are more Hog1 dependent in CWD than in KCl (red, Figure 4.3a). Group II shows the expression components for genes that have similar or less expression in CWD than in KCl (black and green, Figure 4.3a). Genes with no statistically significant component ($p > 0.05$) are left out of Group II. **(D)** CWD specific activation of the MAPK Slt2. Slt2 phosphorylation/activation was assayed by western blotting using antibodies that recognize phosphorylated Slt2 (see Methods). The bar graph shows the average of three replicate experiments, with activation levels normalized to that in CWD. Data for wt cells is shown in black, and *hog1Δ* in gray.

Figure 4.3 (continued)



CWD Specific Activation of Rlm1 by Slt2

How then does Hog1 activate Rlm1 in CWD, where Hog1 is activated at a low level, but not in KCl or glucose stress, where Hog1 is activated at a high level?

Previous studies have shown that the MAPK Slt2, a component of the PKC pathway, phosphorylates and activates Rlm1 in CWD (Jung, et al., 2002; Dodou and Treisman, 1997; Garcia et al., 2004). Therefore, as Rlm1 appears to be activated by both Hog1 and Slt2, it may be the absence of Slt2 activity that prevents Rlm1 activation in high osmolarity. To test for this possibility, we measured the Slt2 activation level in KCl, glucose and CWD by western blotting, using an antibody that recognizes the active, phosphorylated form of the protein. In agreement with previously published reports (Cid et al., 1995; Martin et al., 1996, Bermejo et al., 2008), we find that Slt2 is activated in cell wall damage, but not in KCl or glucose (Figure 4.3d, black bars). By measuring Slt2 phosphorylation in a *hog1Δ* strain, we also established that Slt2 activation in CWD is independent of Hog1 (Figure 4.3d, gray bars), at least under the conditions of our experiment. In contrast, Slt2 activation does depend on Hog1 (genetically) in more moderate stress conditions, but this activation does not seem to strictly require Hog1 kinase activity (Bermejo et al., 2008). Therefore, it seems likely that Rlm1 is integrated into the Hog1 dependent transcriptional network in an Slt2-dependent manner. However, we were not able to study the influence that Slt2 has on gene expression under the conditions used here, due to a growth defect in the *slt2Δ* strain (see Methods), and thus further experiments will be needed to confirm this model.

Low Level Hog1 Activity is transmitted through Sko1 and Hot1

To determine how Hog1 activates genes through factors other than Rlm1 in CWD (e.g. Group IIa, Figure 4.3c), we compared the influence of Hog1, without Rlm1, in CWD (H component, Figure 4.3b) to the influence of Hog1 in KCl, where Rlm1 is inactive. We find that Hog1 activates most genes, through factors other than Rlm1, to a much lower level in CWD than in KCl (Figure 4.4a), consistent with our finding that Hog1 activity is low in CWD. However, some genes are still significantly activated in CWD, a few to a level at or approaching that found in KCl (Figure 4.4a). To determine if the genes activated in CWD are regulated by a particular TF, or combination of TFs, we searched for a correlation between the TF activities assigned in KCl (Capaldi et al., 2008) and the activation levels in CWD. We found two such correlations: the gene-set activated by Hot1 (in KCl), and the gene set repressed by Sko1 (in YEPD), are both highly activated in CWD (red points, Figure 4.4a). In fact, 21 of the 23 genes that are most induced in CWD are activated by Hot1 and/or derepressed by Sko1 in KCl by 4-fold or more. By contrast, only five genes with a low expression level in CWD are activated by Sko1 derpression/Hot1 activation at this cut-off.

We hypothesized that Sko1 and Hot1 might be responsible for activating these genes in CWD, as in KCl. We determined whether the expression of these genes depend on the presence of Sko1 and Hot1 during CWD, again using DNA microarrays (see legend Figure 4.4). As expected, we found significant Sko1

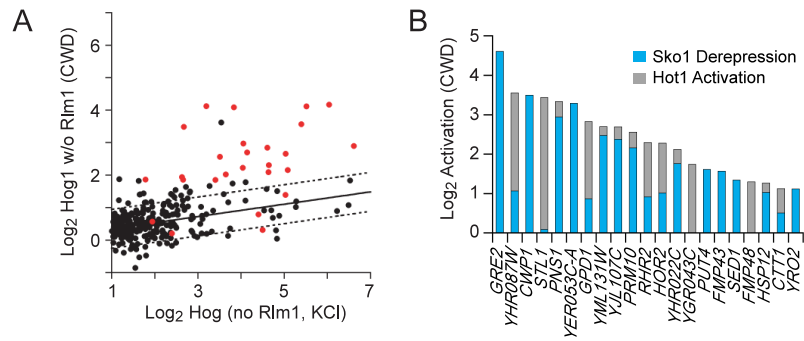


Figure 4.4. Mechanism of Hog1 dependent gene activation in CWD. **(A)** Gene induction by Hog1 in CWD without Rlm1. The influence of Hog1 in KCl and CWD, independent of Rlm1, is compared for the genes from Figs 4.2c and 4.2d. For CWD, this is the H component from the mutant cycle dissection shown in Figure 4.3b. For KCl, the data is the H+Co components from the mutant cycle analysis in Capaldi et al., 2008., since Rlm1 is inactive in these conditions (data in accompanying paper). Genes that are activated by Hot1 and/or derepressed by Sko1 (>4-fold total) in KCl (redundant or otherwise) are shown in red. The solid line shows the best fit to all the other points (black), with a slope of 0.19; demonstrating the low level of TF activation in CWD. The broken lines show the value from the best-fit ± 1.5 -fold change. **(B)** Sko1 is derepressed and Hot1 is activated in CWD. The influence of Sko1 derepression (grey) and Hot1 activation (blue) on gene expression in CWD is shown for all of the genes that are highly activated in CWD (21 red genes above the upper broken line in part A). Sko1 derepression was calculated by comparing the repression due to Sko1 in YEPD (in the absence of Hog1, to match the conditions in part A) to the repression due to Sko1 in CWD. Specifically, repression in YEPD was measured by comparing the expression in *sko1Δhog1Δ* in YEPD to *hog1Δ* in YEPD (genes induced in this array are repressed by Sko1, as described in the accompanying paper). Sko1 repression in CWD was measured by comparing *sko1Δ* in CWD to wt in CWD. We find that Sko1 is inactive in CWD and therefore the level of derepression in CWD is equal to the level of repression in YEPD (these numbers were corrected for any low level repression in CWD). The level of activation from Hot1 was calculated by comparing the expression of *hot1Δ* in CWD to the wt in CWD. The Sko1 and Hot1 data are listed in Table 4.S1.

derepression and Hot1 activation in CWD (Table 4.S1), with a total induction of more than two-fold for all of the genes highly activated in CWD (21 highly expressed red genes, Figure 4.4a) and more than four-fold for 13/21 of these genes (Figure 4.4b). This induction accounts for most of the Hog1-dependent activation at these genes (85% on average, Table 4.S1). Together, these results demonstrate that the low-level Hog1 activity found in CWD is sufficient to allow significant gene induction through Sko1 de-repression and Hot1 activation. By contrast, Sko1 and Msn2/4 activation is very weak in CWD. This suggests that the level of Hog1 activity required to relieve Sko1 repression and activate Hot1 is below that needed to activate Msn2/4 and convert Sko1 into an activator.

Discussion

Taken together our data show that the Hog1 transcriptional network is remarkably flexible, activating different gene expression programs in each of the stress conditions examined. This flexibility is made possible by the distributed structure of the network. In both osmotic shock and CWD, the signal from Hog1 is transmitted through multiple transcription factors, and then recombined in different ways at different genes. However, the strength of the signal transmitted through each factor (and each factor's relative influence) varies from condition to condition (Figure 4.5). As a result, Hog1 activation leads to different, but related, gene expression programs depending on the context.

In high salt stress (KCl), the gene expression program activated by Hog1 involves high-level induction of the general stress response (through Msn2/4) as well as a partially overlapping hyper-osmotic stress specific response (through Sko1 and Hot1) involving processes such as glycerol synthesis and cell wall repair (Capaldi et al., 2008). In CWD, the response is shifted further to focus on cell wall repair genes. This occurs in two ways. First, Sko1 and Msn2/4 activity is dramatically reduced in CWD, and as a result, Hog1 activates 280 fewer osmotic stress genes in CWD than in KCl). Second, Rlm1 is incorporated into the network through an interaction with Hog1 (Figure 4.3) and activates cell wall repair and Hog1/PKC signaling genes.

Beyond establishing that a distributed network structure can lead to a flexible output, the data presented here provides insight into the mechanisms that

underlie the stress-type specific changes in gene expression. Our data suggest that external signals alter the output of the Hog1 transcriptional network by modulating the activity of Rlm1 (Figure 4.5a). It also appears that Hog1 can induce different gene-sets depending on its activity level, since each TF has a different threshold for activation (Figure 4.5b). This model has important implications for the function of transcriptional networks in general. By activating a common set of transcription factors, p38 and other kinases could function together to create a vast number of condition specific gene expression programs. Further analysis of the Hog1 and other transcriptional networks is needed to see if this is the case.

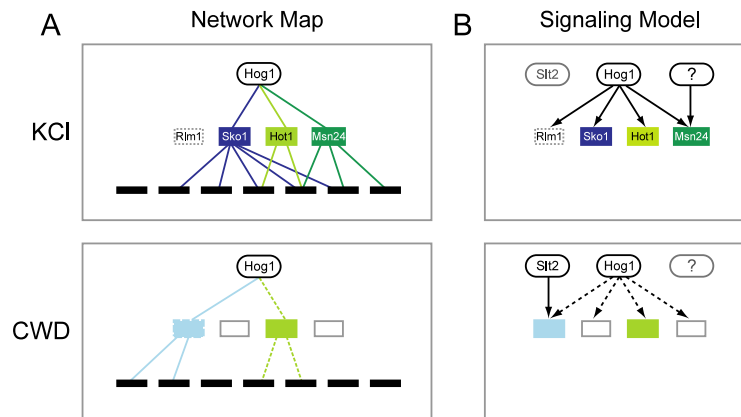


Figure 4.5. Simplified model of stress-specific gene activation by Hog1. **(A)** Structure of the Hog1 network in two different stress conditions. Diagrams show the Hog1 network activated by KCl and CWD (as labeled), highlighting the stress-specific differences in signal transmission. The solid black lines show the regulated genes; grouped by regulatory mechanism. Each box represents one of the Hog1 dependent transcription factors, as labeled in the KCl model. A solid box with solid lines indicates the maximum level of signal transmission through a factor; an empty box with broken lines indicates reduced signal transmission through a factor; and a grey box with no line indicates an inactive TF. **(B)** Model describing stress-type specific gene activation at the signaling level. The solid, open and grey boxes match those in A. Here a solid black arrow represents full kinase activity and a broken line arrow represents partial activity.

In CWD, Hog1 influences the activity of Sko1 and Hot1 even without substantial Hog1 accumulation in the nucleus. In contrast, in osmotic shock, Hog1 is localized to the nucleus in bulk, where it co-binds with its cognate TFs Hot1 and Sko1 at promoters (Alepuz et al., 2001; Alepuz et al., 2003; Proft and Struhl, 2002) and interacts directly with RNA Pol II during elongation (Proft et al., 2006). Hot1 has a small number of targets, and appears to require co-bound Hog1 for activity (Alepuz et al., 2003), while Sko1 influences a larger group of genes, and is regulated by Hog1 phosphorylation in addition to co-binding with Hog1 and Hot1 at promoters (discussed in Chapter 2). In CWD, Hot1 target genes are expressed, but to a lower level than in KCl (Hot1 is responsible for up to 10-fold induction of target genes in CWD, compared to over 100 fold induction in KCl). Perhaps even low levels of nuclear Hog1 are sufficient to co-bind with Hot1 at some promoters to activate gene expression. Alternatively, while Hog1 phosphorylation of Hot1 is not required for gene activation in KCl (Alepuz, 2001), phosphorylation of Hot1 may be sufficient to induce gene expression.

Presumably a small amount of active, nuclear Hog1 is capable of phosphorylating Sko1 and Hot1 in response to stress. Phosphorylation of Sko1 should be sufficient to inhibit the repressive activity of Sko1. However, phosphorylation by Hog1 does not affect Sko1 recruitment to activation targets (Discussed in Chapter 2, Figure 2.3.), which may explain why we do not observe Sko1-dependent activation CWD, when nuclear levels of Hog1 are low. The condition-specific differences in Hog1 activation of Sko1 and Hot1 may provide an

example of stoichiometric (in KCl) versus catalytic (in CWD) mechanisms of Hog1 activity.

Overall, this study points to an important role for signal integration in gene regulation. In our model, the Hog1 transcriptional network does not function as an isolated module, as previously thought (Hohmann, 2002), but rather is regulated by multiple signaling pathways. It is particularly interesting that much of the signal integration we detect appears to occur at the level of TF activation and not at the promoter level through combinatorial control. Signal integration at the level of TF activation cannot be detected/analyzed by genome-wide ChIP assays or by studying gene expression in a single condition. Instead, it requires quantitative comparison and dissection of the influence that signals have on gene expression in a range of conditions, as outlined here. Hence it is possible that much of the signal integration that occurs in transcriptional networks has been overlooked, since few studies have examined input/output relationships in such detail. It will therefore be interesting to apply the approach outlined here to other networks to gain a realistic picture of the role that combinatorial control of TF activity plays in gene regulation.

Materials and Methods

Strains

All strains used in this study are in the W303 strain background (*trp1 leu2 ura3 his3 can1 GAL+ psi+*), and are listed in Table 4.1. Gene deletions were introduced by transformation with PCR products including auxotrophic, or antibiotic resistance, markers flanked by the 40 bp sequence found directly upstream and downstream from the gene, followed by selection on the appropriate media (Rothstein, 1991). Strains with multiple gene KOs were constructed by mating the single delete strains and dissecting the resulting tetrads. Tagged strains (GFP) were constructed in a similar way (Longtine et al., 1998) but the primers directed the recombination to the sites directly upstream and directly downstream from the appropriate stop codon. Insertion of the GFP tag into the correct locus was confirmed by PCR.

Strain	Genotype	Background
EY0690	w303 MATa	EY0690
<i>hog1Δ</i>	<i>hog1::Ura</i> MATa	EY0690
<i>hot1Δ</i>	<i>hot1::His3</i> MATa	EY0690
<i>rlm1Δ</i>	<i>rlm1::HIS3</i> MATa	EY0690
<i>sko1Δ</i>	<i>sko1::Leu2</i> MATa	EY0690
<i>smp1Δ</i>	<i>smp1::His3</i> MATa	EY0690
<i>slt2Δ</i>	<i>slt2::HIS3</i> MATa	EY0690
<i>hog1Δrlm1Δ</i>	<i>hog1::URA3, rlm1::HIS3</i> MATa	EY0690
<i>msn2Δ msn4Δ</i>	<i>msn2::Leu2 msn4::His3</i> MATa	EY0690
<i>hog1Δ msn2Δ msn4Δ</i>	<i>hog1::Ura msn2::Leu2 msn4::His3</i> MATa	EY0690
Hog1-GFP Nhp6a-RFP	Hog1-GFP(His) Nhp6a-RFP(KanMX6) MATa	EY0690
Msn2-GFP Nhp6a-RFP	Msn2-GFP(His) Nhp6a-RFP(KanMX6) MATa	EY0690
Nhp6a-RFP	Nhp6a-RFP(KanMX6) MATα	EY0691

Table 4.1. List of strains used in this study.

Microarrays

Microarrays and data analysis were carried out as described previously (Capaldi et al., 2008) with one exception. To remove cell wall contaminants from the RNA of zymolyase treated cells, we added an additional centrifugation step (12,000 rpm for 40 minutes) after the phenol/chloroform extraction, and precipitated the RNA from the supernatant. We could not analyze the influence that Slt2 has on gene expression in zymolyase since *slt2Δ* cells are temperature sensitive, and zymolyase activity is greatly reduced at the permissive growth temperature.

The KCl, high glucose and CWD data was measured after 20 min of stress. Preliminary experiments using home printed arrays (data not shown) showed that

Hog1-dependent expression reaches a maximum at this time-point, in all three conditions. By measuring expression at this early time-point we avoid detecting secondary effects on gene expression. We used 5U/ml zymolyase in our CWD experiments since this is the highest concentration of enzyme that does not cause significant lysis in the *hog1* Δ strain (*hog1* Δ was more sensitive to zymolyase than the wt strain).

Microscopy

Microscopy was carried out as described by Capaldi et al. (2008), and these experiments were performed by Ying Liu. Details specific to the cell wall damage experiment are described here. Strains expressing a GFP tagged protein and RFP-tagged Nhp6a (a nuclear marker), were grown in SD medium to an OD₆₀₀ of 0.1 at 30 °C. These tubes were then grown at room temperature (23 °C) with shaking for approx 1 hr. 50 μ l of cells were then added to a well of a 96 well glass bottomed plate and allowed to settle for 5-10 min before treatment with 5 units per mL zymolyase (Zymoresearch). As common fixing agents can activate the cell wall integrity pathway, we avoided fixing the cells. The values reported are the average and standard deviation from three biological replicate experiments.

Western blots

Cultures were grown to mid-log phase in YEPD medium and then subjected to stress (0.375 M KCl, 0.750 M glucose, or 5 units/mL zymolyase). After 15 min cells were harvested and lysed by bead beating for 5 min at 100 °C in 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 1 mM DTT, 2 mM PMSF, 2.5 mM benzamidine, 1 μ g/mL

leupeptin, 1 µg/mL pepstatin, 80 mM β-glycerophosphate, 10 mM NaF, 20 nM calyculin A. 150µl of each sample was then transferred to a tube containing an equal volume of high salt buffer buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% NP40, 1 mM EDTA, 10% glycerol, 1 mM DTT, 2 mM PMSF, 2.5 mM benzamidine, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 80 mM β-glycerophosphate, 10 mM NaF, 20 nM calyculin A), and centrifuged for 10 min at 14,000 rpm. The supernatant was then run on a 10% SDS polyacrylamide gel, transferred to a nitrocellulose membrane and analyzed using anti-phospho-p44/42 MAP Kinase antibody (Cell Signaling Technology) at a 1:250 dilution.

List of supplemental data

Table 4.S1 Array data from experiments in cell wall damage.

Table 4.S2 Components for all yeast genes from quantitative epistasis analysis of Hog1 and Msn2/4 (shown in Figure 4.2).

Table 4.S3 Components for all yeast genes from quantitative epistasis analysis of Hog1 and Rlm1 (shown in Figure 4.3B-C).

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Chapter 5

Summary and Future Directions

Summary

The goal of this chapter is to first present an overall picture of the work described in the previous chapters, and then identify further questions that could be the focus of follow up work. Transcriptional responses to environmental changes, such as osmotic shock, temperature shift, or oxidative damage, coordinate the induction of hundreds of genes within minutes or tens of minutes, increasing their expression levels by up to two orders of magnitude in (Gasch et al., 2000). In addition, these responses rapidly induce the production of hundreds of copies of individual mRNAs, while other stress-induced genes are expressed at a more moderate level. If rapidly dividing yeast cells are operating close to their maximal transcriptional capacity while growing in rich medium, it may not be possible to add the stress response to the existing transcriptional program. To ensure a rapid transcriptional response, cells may need to temporarily divert some transcriptional resources away from the rapid growth transcription program.

Recent work suggests that during osmotic shock, the overall transcription rate decreases temporarily (Romero-Santacreu, et al., 2009; Miller, et al., 2011). This decrease in global decrease in transcription occurs within the first few minutes of stress exposure, when physical effects of stress appear to compromise the binding of both general and specific transcription factors to DNA (Proft and Struhl 2004), compromising the cell's ability to initiate transcription. However, rapid induction of stress responsive genes occurs within this same window of time (Miller et al., 2011). We find that cell prioritizes transcription of stress responsive genes

over housekeeping genes during the initial shock phase of the stress response. During the induction phase of the transcriptional stress response, RNA Pol II is depleted from genes that were highly expressed prior to stress, and simultaneously recruited to stress-induced genes. We demonstrate that depletion of RNA Pol II from housekeeping genes upon stress is a general effect, rather than a gene-specific behavior. This implies that stress-induced genes are somehow exempted from this global effect. Induction of these genes during stress shows genetic dependence on the MAP kinase Hog1. We find that the majority of genes that are highly expressed during stress are selectively transcribed by a Hog1-RNA Pol II complex. We suggest that the ability of genes to recruit or assemble this complex could allow induction of stress responsive genes despite a global decrease in transcription.

Two previously identified Hog1 cognate transcription factors, Sko1 and Hot1, co-localize with Hog1 in the promoters of genes that are transcribed by the Hog1-RNA Pol II complex (Alepuz et al., 2001; Alepuz et al., 2003; Proft and Struhl, 2002). Predicted binding sites for these two factors occur in closely spaced pairs (< 50 base pairs apart). For both of these transcription factors, promoter recognition requires Hog1, but does not depend on phosphorylation by Hog1. Instead, Hog1 appears to act as a binding co-factor. Both of these factors are found in the nucleus in the presence and absence of stress, but they are recruited to Hog1-RNA Pol II target genes only in the presence of stress. In cells lacking Sko1 and Hot1, these target genes are no longer selectively transcribed by the Hog1-RNA Pol II complex. In stress, Sko1 and Hot1 recognize low information binding sites at target genes with the help of Hog1. Although both Sko1 and Hot1 are present in the nucleus in the

absence of stress, they do not recognize the low information sites in the promoters of target genes under favorable growth conditions. The entry of Hog1 into the nucleus serves as a signal to induce occupancy of these sites.

Remaining questions

While Hot1 and Sko1 binding sites are found in closely spaced pairs, it is not clear if these factors can influence one another's binding behavior in some way. It would be interesting to know if Sko1 and Hot1 show cooperative binding at the promoters of target genes, and if recruitment of the Hog1-Pol II complex to target genes requires the presence of both of these sites.

It is not clear whether the Hog1-RNA Pol II complex assembles at target promoters, or if it can also exist free of DNA. If Hog1 is in complex with RNA Pol II free of DNA, it seems possible that the existence of this complex could have global effects on transcription. For example, perhaps the Hog1 Pol II complex cannot efficiently initiate transcription at promoters lacking the Hog1 cognate factors Sko1 and Hot1. In this scenario, Hog1 commandeers some portion of the pool of RNA Pol II, such that stress responsive genes are transcribed by a dedicated pool of polymerase. It is also possible that Hog1 phosphorylates RNA Pol II, or some component of the general transcription machinery, affecting its activity or selectivity in some way.

It is clear that the Hog1-RNA Pol II complex that forms in the initial stages of the *S. cerevisiae* response to osmotic shock is involved in the rapid production of hundreds of mRNAs of individual target genes. In this sense, the Hog1-RNA Pol II complex plays a role in redistributing RNA Pol II upon stress. However, it is not clear if the formation of a Hog1-RNA Pol II complex is related to the global decrease in transcription upon stress. The decrease appears to be partially dependent on Hog1 (Romero-Santacreu et al., 2009), but Hog1 may impact global transcription through some other mechanism that does not require formation of a Hog1-RNA Pol II complex. However, given that Hog1 is known to interact directly with general transcription machinery, it would be interesting to know if this complex has a role in regulating global transcription rates.

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